

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
6 June 2002 (06.06.2002)

PCT

(10) International Publication Number
WO 02/44321 A2

- (51) International Patent Classification⁷: C12N (74) Agent: WEICKMANN & WEICKMANN; Postfach 860
820, 81635 München (DE).
- (21) International Application Number: PCT/EP01/13968
- (22) International Filing Date:
29 November 2001 (29.11.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
00126325.0 1 December 2000 (01.12.2000) EP
60/279,661 30 March 2001 (30.03.2001) US
PCT/US01/10188 30 March 2001 (30.03.2001) US
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- (71) Applicant (*for all designated States except US*): MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE]; Hofgartenstrasse 2, 80539 München (DE).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): TUSCHL, Thomas [DE/DE]; Keplerstrasse 9, 37085 Göttingen (DE). EL-BASHIR, Sayda [DE/DE]; Sultebecksbreite 2, 37075 Göttingen (DE). LENDECKEL, Winfried [DE/DE]; Blinde Gasse 52, 37318 Hohengandern (DE).
- Published:
— *without international search report and to be republished upon receipt of that report*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 02/44321 A2

(54) Title: RNA INTERFERENCE MEDIATING SMALL RNA MOLECULES

(57) Abstract: Double-stranded RNA (dsRNA) induces sequence-specific post-transcriptional gene silencing in many organisms by a process known as RNA interference (RNAi). Using a *Drosophila* in vitro system, we demonstrate that 19-23 nt short RNA fragments are the sequence-specific mediators of RNAi. The short interfering RNAs (siRNAs) are generated by an RNase III-like processing reaction from long dsRNA. Chemically synthesized siRNA duplexes with overhanging 3' ends mediate efficient target RNA cleavage in the lysate, and the cleavage site is located near the center of the region spanned by the guiding siRNA. Furthermore, we provide evidence that the direction of dsRNA processing determines whether sense or antisense target RNA can be cleaved by the produced siRNP complex.

11/22/04, EAST Version: 2.0.1.4

BEST AVAILABLE COPY

RNA Interference Mediating Small RNA molecules

Description

5

The present invention relates to sequence and structural features of double-stranded (ds)RNA molecules required to mediate target-specific nucleic acid modifications such as RNA-interference and/or DNA methylation.

10

The term "RNA interference" (RNAi) was coined after the discovery that injection of dsRNA into the nematode *C. elegans* leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA (Fire et al., 1998). RNAi was subsequently also observed in insects, frogs (Oelgeschlager et al., 2000), and other animals including mice (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000) and is likely to also exist in human. RNAi is closely linked to the post-transcriptional gene-silencing (PTGS) mechanism of co-suppression in plants and quelling in fungi (Catalanotto et al., 2000; Cogoni and Macino, 1999; Dalmay et al., 2000; Ketting and Plasterk, 2000; Mourrain et al., 2000; Smardon et al., 2000) and some components of the RNAi machinery are also necessary for post-transcriptional silencing by co-suppression (Catalanotto et al., 2000; Dernburg et al., 2000; Ketting and Plasterk, 2000). The topic has also been reviewed recently (Bass, 2000; Boshier and Labouesse, 2000; Fire, 1999; Plasterk and Ketting, 2000; Sharp, 1999; Sijen and Kooter, 2000), see also the entire issue of Plant Molecular Biology, vol. 43, issue 2/3, (2000).

In plants, in addition to PTGS, introduced transgenes can also lead to transcriptional gene silencing via RNA-directed DNA methylation of cytosines (see references in Wassenegger, 2000). Genomic targets as short as 30 bp are methylated in plants in an RNA-directed manner (Pelissier, 2000). DNA methylation is also present in mammals.

30

- 2 -

The natural function of RNAi and co-suppression appears to be protection of the genome against invasion by mobile genetic elements such as retro-transposons and viruses which produce aberrant RNA or dsRNA in the host cell when they become active (Jensen et al, 1999; Ketting et al., 1999; Ratcliff et al., 1999; Tabara et al., 1999). Specific mRNA degradation prevents transposon and virus replication although some viruses are able to overcome or prevent this process by expressing proteins that suppress PTGS (Lucy et al., 2000; Voinnet et al., 2000).

DsRNA triggers the specific degradation of homologous RNAs only within the region of identity with the dsRNA (Zamore et al., 2000). The dsRNA is processed to 21-23 nt RNA fragments and the target RNA cleavage sites are regularly spaced 21-23 nt apart. It has therefore been suggested that the 21-23 nt fragments are the guide RNAs for target recognition (Zamore et al., 2000). These short RNAs were also detected in extracts prepared from *D. melanogaster* Schneider 2 cells which were transfected with dsRNA prior to cell lysis (Hammond et al., 2000), however, the fractions that displayed sequence-specific nuclease activity also contained a large fraction of residual dsRNA. The role of the 21-23 nt fragments in guiding mRNA cleavage is further supported by the observation that 21-23 nt fragments isolated from processed dsRNA are able, to some extent, to mediate specific mRNA degradation (Zamore et al., 2000). RNA molecules of similar size also accumulate in plant tissue that exhibits PTGS (Hamilton and Baulcombe, 1999).

Here, we use the established *Drosophila* in vitro system (Tuschl et al., 1999; Zamore et al., 2000) to further explore the mechanism of RNAi. We demonstrate that short 21 and 22 nt RNAs, when base-paired with 3' overhanging ends, act as the guide RNAs for sequence-specific mRNA degradation. Short 30 bp dsRNAs are unable to mediate RNAi in this system because they are no longer processed to 21 and 22 nt RNAs. Furthermore, we defined the target RNA cleavage sites relative to the 21 and

- 3 -

22 nt short interfering RNAs (siRNAs) and provide evidence that the direction of dsRNA processing determines whether a sense or an antisense target RNA can be cleaved by the produced siRNP endonuclease complex. Further, the siRNAs may also be important tools for transcriptional modulating, e.g. silencing of mammalian genes by guiding DNA methylation.

Further experiments in human in vivo cell culture systems (HeLa cells) show that double-stranded RNA molecules having a length of preferably from 19-25 nucleotides have RNAi activity. Thus, in contrast to the results from *Drosophila* also 24 and 25 nt long double-stranded RNA molecules are efficient for RNAi.

The object underlying the present invention is to provide novel agents capable of mediating target-specific RNA interference or other target-specific nucleic acid modifications such as DNA methylation, said agents having an improved efficacy and safety compared to prior art agents.

The solution of this problem is provided by an isolated double-stranded RNA molecule, wherein each RNA strand has a length from 19-25, particularly from 19-23 nucleotides, wherein said RNA molecule is capable of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation. Preferably at least one strand has a 3'-overhang from 1-5 nucleotides, more preferably from 1-3 nucleotides and most preferably 2 nucleotides. The other strand may be blunt-ended or has up to 6 nucleotides 3' overhang. Also, if both strands of the dsRNA are exactly 21 or 22 nt, it is possible to observe some RNA interference when both ends are blunt (0 nt overhang). The RNA molecule is preferably a synthetic RNA molecule which is substantially free from contaminants occurring in cell extracts, e.g. from *Drosophila* embryos. Further, the RNA molecule is preferably substantially free from any non-target-specific contaminants, particularly non-target-specific RNA molecules e.g. from contaminants occurring in cell extracts.

- 4 -

Further, the invention relates to the use of isolated double-stranded RNA molecules, wherein each RNA strand has a length from 19-25 nucleotides, for mediating, target-specific nucleic acid modifications, particularly RNAi, in mammalian cells, particularly in human cells.

5

Surprisingly, it was found that synthetic short double-stranded RNA molecules particularly with overhanging 3'-ends are sequence-specific mediators of RNAi and mediate efficient target-RNA cleavage, wherein the cleavage site is located near the center of the region spanned by the guiding short RNA.

10

Preferably, each strand of the RNA molecule has a length from 20-22 nucleotides (or 20-25 nucleotides in mammalian cells), wherein the length of each strand may be the same or different. Preferably, the length of the 3'-overhang reaches from 1-3 nucleotides, wherein the length of the overhang may be the same or different for each strand. The RNA-strands preferably have 3'-hydroxyl groups. The 5'-terminus preferably comprises a phosphate, diphosphate, triphosphate or hydroxyl group. The most effective dsRNAs are composed of two 21 nt strands which are paired such that 1-3, particularly 2 nt 3' overhangs are present on both ends of the dsRNA.

15

20

The target RNA cleavage reaction guided by siRNAs is highly sequence-specific. However, not all positions of a siRNA contribute equally to target recognition. Mismatches in the center of the siRNA duplex are most critical and essentially abolish target RNA cleavage. In contrast, the 3' nucleotide of the siRNA strand (e.g. position 21) that is complementary to the single-stranded target RNA, does not contribute to specificity of the target recognition. Further, the sequence of the unpaired 2-nt 3' overhang of the siRNA strand with the same polarity as the target RNA is not critical for target RNA cleavage as only the antisense siRNA strand guides target recognition. Thus, from the single-stranded overhanging nucleotides only the

25

30

penultimate position of the antisense siRNA (e.g. position 20) needs to match the targeted sense mRNA.

Surprisingly, the double-stranded RNA molecules of the present invention exhibit a high in vivo stability in serum or in growth medium for cell cultures. In order to further enhance the stability, the 3'-overhangs may be stabilized against degradation, e.g. they may be selected such that they consist of purine nucleotides, particularly adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g. substitution of uridine 2 nt 3' overhangs by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNA interference. The absence of a 2' hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium.

In an especially preferred embodiment of the present invention the RNA molecule may contain at least one modified nucleotide analogue. The nucleotide analogues may be located at positions where the target-specific activity, e.g. the RNAi mediating activity is not substantially effected, e.g. in a region at the 5'-end and/or the 3'-end of the double-stranded RNA molecule. Particularly, the overhangs may be stabilized by incorporating modified nucleotide analogues.

Preferred nucleotide analogues are selected from sugar- or backbone-modified ribonucleotides. It should be noted, however, that also nucleobase-modified ribonucleotides, i.e. ribonucleotides, containing a non-naturally occurring nucleobase instead of a naturally occurring nucleobase such as uridines or cytidines modified at the 5-position, e.g. 5-(2-amino)propyl uridine, 5-bromo uridine; adenosines and guanosines modified at the 8-position, e.g. 8-bromo guanosine; deaza nucleotides, e.g. 7-deaza-adenosine; O- and N-alkylated nucleotides, e.g. N6-methyl adenosine are suitable. In preferred sugar-modified ribonucleotides the 2' OH-group is repla-

- 6 -

ced by a group selected from H, OR, R, halo, SH, SR, NH₂, NHR, NR₂ or CN, wherein R is C₁-C₆ alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I. In preferred backbone-modified ribonucleotides the phosphoester group connecting to adjacent ribonucleotides is replaced by a modified group, e.g. of phosphothioate group. It should be noted that the above modifications may be combined.

The sequence of the double-stranded RNA molecule of the present invention has to have a sufficient identity to a nucleic acid target molecule in order to mediate target-specific RNAi and/or DNA methylation. Preferably, the sequence has an identity of at least 50%, particularly of at least 70% to the desired target molecule in the double-stranded portion of the RNA molecule. More preferably, the identity is at least 85% and most preferably 100% in the double-stranded portion of the RNA molecule. The identity of a double-stranded RNA molecule to a predetermined nucleic acid target molecule, e.g. an mRNA target molecule may be determined as follows:

$$I = \frac{n}{L} \times 100$$

wherein I is the identity in percent, n is the number of identical nucleotides in the double-stranded portion of the ds RNA and the target and L is the length of the sequence overlap of the double-stranded portion of the dsRNA and the target.

Alternatively, the identity of the double-stranded RNA molecule to the target sequence may also be defined including the 3' overhang, particularly an overhang having a length from 1-3 nucleotides. In this case the sequence identity is preferably at least 50%, more preferably at least 70% and most preferably at least 85% to the target sequence. For example, the nucleotides from the 3' overhang and up to 2 nucleotides from the 5' and/or 3' terminus of the double strand may be modified without significant loss of activity.

- 7 -

The double-stranded RNA molecule of the invention may be prepared by a method comprising the steps:

- 5 (a) synthesizing two RNA strands each having a length from 19-25, e.g. from 19-23 nucleotides, wherein said RNA strands are capable of forming a double-stranded RNA molecule, wherein preferably at least one strand has a 3'-overhang from 1-5 nucleotides,
- 10 (b) combining the synthesized RNA strands under conditions, wherein a double-stranded RNA molecule is formed, which is capable of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation.

15 Methods of synthesizing RNA molecules are known in the art. In this context, it is particularly referred to chemical synthesis methods as described in Verma and Eckstein (1998).

20 The single-stranded RNAs can also be prepared by enzymatic transcription from synthetic DNA templates or from DNA plasmids isolated from recombinant bacteria. Typically, phage RNA polymerases are used such as T7, T3 or SP6 RNA polymerase (Milligan and Uhlenbeck (1989)).

25 A further aspect of the present invention relates to a method of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation in a cell or an organism comprising the steps:

- 30 (a) contacting the cell or organism with the double-stranded RNA molecule of the invention under conditions wherein target-specific nucleic acid modifications may occur and
- (b) mediating a target-specific nucleic acid modification effected by the double-stranded RNA towards a target nucleic acid having a

- 8 -

sequence portion substantially corresponding to the double-stranded RNA.

5 Preferably the contacting step (a) comprises introducing the double-stranded RNA molecule into a target cell, e.g. an isolated target cell, e.g. in cell culture, a unicellular microorganism or a target cell or a plurality of target cells within a multicellular organism. More preferably, the introducing step comprises a carrier-mediated delivery, e.g. by liposomal carriers or by injection.

10

The method of the invention may be used for determining the function of a gene in a cell or an organism or even for modulating the function of a gene in a cell or an organism, being capable of mediating RNA interference. The cell is preferably a eukaryotic cell or a cell line, e.g. a plant cell or an animal cell, such as a mammalian cell, e.g. an embryonic cell, a pluripotent stem cell, a tumor cell, e.g. a teratocarcinoma cell or a virus-infected cell. 15 The organism is preferably a eukaryotic organism, e.g. a plant or an animal, such as a mammal, particularly a human.

20 The target gene to which the RNA molecule of the invention is directed may be associated with a pathological condition. For example, the gene may be a pathogen-associated gene, e.g. a viral gene, a tumor-associated gene or an autoimmune disease-associated gene. The target gene may also be a heterologous gene expressed in a recombinant cell or a genetically altered organism. By determining or modulating, particularly, inhibiting 25 the function of such a gene valuable information and therapeutic benefits in the agricultural field or in the medicine or veterinary medicine field may be obtained.

30 The dsRNA is usually administered as a pharmaceutical composition. The administration may be carried out by known methods, wherein a nucleic acid is introduced into a desired target cell in vitro or in vivo. Commonly

used gene transfer techniques include calcium phosphate, DEAE-dextran, electroporation and microinjection and viral methods (Graham, F.L. and van der Eb, A.J. (1973) *Viol.* 52, 456; McCutchan, J.H. and Pagano, J.S. (1968), *J. Natl. Cancer Inst.* 41, 351; Chu, G. et al (1987), *Nucl. Acids Res.* 15, 1311; Fraley, R. et al. (1980), *J. Biol. Chem.* 255, 10431; Capecchi, M.R. (1980), *Cell* 22, 479). A recent addition to this arsenal of techniques for the introduction of DNA into cells is the use of cationic liposomes (Felgner, P.L. et al. (1987), *Proc. Natl. Acad. Sci USA* 84, 7413). Commercially available cationic lipid formulations are e.g. Tfx 50 (Promega) or Lipofectamin2000 (Life Technologies).

Thus, the invention also relates to a pharmaceutical composition containing as an active agent at least one double-stranded RNA molecule as described above and a pharmaceutical carrier. The composition may be used for diagnostic and for therapeutic applications in human medicine or in veterinary medicine.

For diagnostic or therapeutic applications, the composition may be in form of a solution, e.g. an injectable solution, a cream, ointment, tablet, suspension or the like. The composition may be administered in any suitable way, e.g. by injection, by oral, topical, nasal, rectal application etc. The carrier may be any suitable pharmaceutical carrier. Preferably, a carrier is used, which is capable of increasing the efficacy of the RNA molecules to enter the target-cells. Suitable examples of such carriers are liposomes, particularly cationic liposomes. A further preferred administration method is injection.

A further preferred application of the RNAi method is a functional analysis of eukaryotic cells, or eukaryotic non-human organisms, preferably mammalian cells or organisms and most preferably human cells, e.g. cell lines such as HeLa or 293 or rodents, e.g. rats and mice. By transfection with suitable double-stranded RNA molecules which are homologous to a prede-

- 10 -

terminated target gene or DNA molecules encoding a suitable double-stranded RNA molecule a specific knockout phenotype can be obtained in a target cell, e.g. in cell culture or in a target organism. Surprisingly it was found that the presence of short double-stranded RNA molecules does not
5 result in an interferon response from the host cell or host organism.

Thus, a further subject matter of the invention is a eukaryotic cell or a eukaryotic non-human organism exhibiting a target gene-specific knockout phenotype comprising an at least partially deficient expression of at least
10 one endogenous target gene wherein said cell or organism is transfected with at least one double-stranded RNA molecule capable of inhibiting the expression of at least one endogenous target gene or with a DNA encoding at least one double stranded RNA molecule capable of inhibiting the expression of at least one endogenous target gene. It should be noted
15 that the present invention allows a target-specific knockout of several different endogenous genes due to the specificity of RNAi.

Gene-specific knockout phenotypes of cells or non-human organisms, particularly of human cells or non-human mammals may be used in analytic
20 procedures, e.g. in the functional and/or phenotypical analysis of complex physiological processes such as analysis of gene expression profiles and/or proteomes. For example, one may prepare the knock-out phenotypes of human genes in cultured cells which are assumed to be regulators of alternative splicing processes. Among these genes are particularly the
25 members of the SR splicing factor family, e.g. ASF/SF2, SC35, SRp20, SRp40 or SRp55. Further, the effect of SR proteins on the mRNA profiles of predetermined alternatively spliced genes such as CD44 may be analysed. Preferably the analysis is carried out by high-throughput methods using oligonucleotide based chips.

30

Using RNAi based knockout technologies, the expression of an endogenous target gene may be inhibited in a target cell or a target organism.

- 11 -

The endogeneous gene may be complemented by an exogeneous target nucleic acid coding for the target protein or a variant or mutated form of the target protein, e.g. a gene or a cDNA, which may optionally be fused to a further nucleic acid sequence encoding a detectable peptide or polypeptide, e.g. an affinity tag, particularly a multiple affinity tag. Variants or mutated forms of the target gene differ from the endogeneous target gene in that they encode a gene product which differs from the endogeneous gene product on the amino acid level by substitutions, insertions and/or deletions of single or multiple amino acids. The variants or mutated forms may have the same biological activity as the endogeneous target gene. On the other hand, the variant or mutated target gene may also have a biological activity, which differs from the biological activity of the endogeneous target gene, e.g. a partially deleted activity, a completely deleted activity, an enhanced activity etc.

The complementation may be accomplished by coexpressing the polypeptide encoded by the exogeneous nucleic acid, e.g. a fusion protein comprising the target protein and the affinity tag and the double stranded RNA molecule for knocking out the endogeneous gene in the target cell. This coexpression may be accomplished by using a suitable expression vector expressing both the polypeptide encoded by the exogeneous nucleic acid, e.g. the tag-modified target protein and the double stranded RNA molecule or alternatively by using a combination of expression vectors. Proteins and protein complexes which are synthesized de novo in the target cell will contain the exogeneous gene product, e.g. the modified fusion protein. In order to avoid suppression of the exogeneous gene product expression by the RNAi duplex molecule, the nucleotide sequence encoding the exogeneous nucleic acid may be altered on the DNA level (with or without causing mutations on the amino acid level) in the part of the sequence which is homologous to the double stranded RNA molecule. Alternatively, the endogeneous target gene may be complemented by corresponding nucleotide sequences from other species, e.g. from mouse.

- 12 -

Preferred applications for the cell or organism of the invention is the analysis of gene expression profiles and/or proteomes. In an especially preferred embodiment an analysis of a variant or mutant form of one or several target proteins is carried out, wherein said variant or mutant forms are reintroduced into the cell or organism by an exogenous target nucleic acid as described above. The combination of knockout of an endogenous gene and rescue by using mutated, e.g. partially deleted exogenous target has advantages compared to the use of a knockout cell. Further, this method is particularly suitable for identifying functional domains of the target protein. In a further preferred embodiment a comparison, e.g. of gene expression profiles and/or proteomes and/or phenotypic characteristics of at least two cells or organisms is carried out. These organisms are selected from:

- (i) a control cell or control organism without target gene inhibition,
- (ii) a cell or organism with target gene inhibition and
- (iii) a cell or organism with target gene inhibition plus target gene complementation by an exogenous target nucleic acid.

The method and cell of the invention are also suitable in a procedure for identifying and/or characterizing pharmacological agents, e.g. identifying new pharmacological agents from a collection of test substances and/or characterizing mechanisms of action and/or side effects of known pharmacological agents.

Thus, the present invention also relates to a system for identifying and/or characterizing pharmacological agents acting on at least one target protein comprising:

- (a) a eukaryotic cell or a eukaryotic non-human organism capable of expressing at least one endogenous target gene coding for said target protein,
- (b) at least one double-stranded RNA molecule capable of inhibiting the expression of said at least one endogenous target gene, and

- 13 -

- (c) a test substance or a collection of test substances wherein pharmacological properties of said test substance or said collection are to be identified and/or characterized.

5 Further, the system as described above preferably comprises:

- (d) at least one exogenous target nucleic acid coding for the target protein or a variant or mutated form of the target protein wherein said exogenous target nucleic acid differs from the endogenous target gene on the nucleic acid level such that the expression of the exogenous target nucleic acid is substantially less inhibited by the double stranded RNA molecule than the expression of the endogenous target gene.
- 10

Furthermore, the RNA knockout complementation method may be used for preparative purposes, e.g. for the affinity purification of proteins or protein complexes from eukaryotic cells, particularly mammalian cells and more particularly human cells. In this embodiment of the invention, the exogenous target nucleic acid preferably codes for a target protein which is fused to an affinity tag.

15

20 The preparative method may be employed for the purification of high molecular weight protein complexes which preferably have a mass of ≥ 150 kD and more preferably of ≥ 500 kD and which optionally may contain nucleic acids such as RNA. Specific examples are the heterotrimeric protein complex consisting of the 20 kD, 60 kD and 90 kD proteins of the U4/U6 snRNP particle, the splicing factor SF3b from the 17S U2 snRNP consisting of 5 proteins having molecular weights of 14, 49, 120, 145 and 155 kD and the 25S U4/U6/U5 tri-snRNP particle containing the U4, U5 and U6 snRNA molecules and about 30 proteins, which has a molecular weight of about 1.7 MD.

25

30

- 14 -

This method is suitable for functional proteome analysis in mammalian cells, particularly human cells.

Further, the present invention is explained in more detail in the following
5 figures and examples.

Figure Legends

Figure 1: Double-stranded RNA as short as 38 bp can mediate RNAi.

10 (A) Graphic representation of dsRNAs used for targeting Pp-luc mRNA. Three series of blunt-ended dsRNAs covering a range of 29 to 504 bp were prepared. The position of the first nucleotide of the sense strand of the dsRNA is indicated relative to the start codon of Pp-luc mRNA (p1). (B)
RNA interference assay (Tuschl et al., 1999). Ratios of target Pp-luc to
15 control Rr-luc activity were normalized to a buffer control (black bar). DsRNAs (5 nM) were preincubated in Drosophila lysate for 15 min at 25°C prior to the addition of 7-methyl-guanosine-capped Pp-luc and Rr-luc mRNAs (~50 pM). The incubation was continued for another hour and then analyzed by the dual luciferase assay (Promega). The data are the
20 average from at least four independent experiments \pm standard deviation.

Figure 2: A 29 bp dsRNA is no longer processed to 21-23 nt fragments. Time course of 21-23 mer formation from processing of internally ^{32}P -labeled dsRNAs (5 nM) in the Drosophila lysate. The length and source of
25 the dsRNA are indicated. An RNA size marker (M) has been loaded in the left lane and the fragment sizes are indicated. Double bands at time zero are due to incompletely denatured dsRNA.

Figure 3: Short dsRNAs cleave the mRNA target only once.

30 (A) Denaturing gel electrophoreses of the stable 5' cleavage products produced by 1 h incubation of 10 nM sense or antisense RNA ^{32}P -labeled at the cap with 10 nM dsRNAs of the p133 series in Drosophila lysate.

- 15 -

Length markers were generated by partial nuclease T1 digestion and partial alkaline hydrolysis (OH) of the cap-labeled target RNA. The regions targeted by the dsRNAs are indicated as black bars on both sides. The 20-23 nt spacing between the predominant cleavage sites for the 111 bp long dsRNA is shown. The horizontal arrow indicates unspecific cleavage not due to RNAi. (B) Position of the cleavage sites on sense and antisense target RNAs. The sequences of the capped 177 nt sense and 180 nt antisense target RNAs are represented in antiparallel orientation such that complementary sequence are opposing each other. The region targeted by the different dsRNAs are indicated by differently colored bars positioned between sense and antisense target sequences. Cleavage sites are indicated by circles: large circle for strong cleavage, small circle for weak cleavage. The ³²P-radiolabeled phosphate group is marked by an asterisk.

Figure 4: 21 and 22 nt RNA fragments are generated by an RNase III-like mechanism.

(A) Sequences of ~21 nt RNAs after dsRNA processing. The ~21 nt RNA fragments generated by dsRNA processing were directionally cloned and sequenced. Oligoribonucleotides originating from the sense strand of the dsRNA are indicated as blue lines, those originating from the antisense strand as red lines. Thick bars are used if the same sequence was present in multiple clones, the number at the right indicating the frequency. The target RNA cleavage sites mediated by the dsRNA are indicated as orange circles, large circle for strong cleavage, small circle for weak cleavage (see Figure 3B). Circles on top of the sense strand indicated cleavage sites within the sense target and circles at the bottom of the dsRNA indicate cleavage site in the antisense target. Up to five additional nucleotides were identified in ~21 nt fragments derived from the 3' ends of the dsRNA. These nucleotides are random combinations of predominantly C, G, or A residues and were most likely added in an untemplated fashion during T7 transcription of the dsRNA-constituting strands. (B) Two-dimensional TLC analysis of the nucleotide composition of ~21 nt RNAs. The ~21 nt RNAs

- 16 -

were generated by incubation of internally radiolabeled 504 bp Pp-luc dsRNA in *Drosophila* lysate, gel-purified, and then digested to mononucleotides with nuclease P1 (top row) or ribonuclease T2 (bottom row). The dsRNA was internally radiolabeled by transcription in the presence of one of the indicated α - ^{32}P nucleoside triphosphates. Radioactivity was detected by phosphorimaging. Nucleoside 5'-monophosphates, nucleoside 3'-monophosphates, nucleoside 5',3'-diphosphates, and inorganic phosphate are indicated as pN, Np, pNp, and p_i, respectively. Black circles indicate UV-absorbing spots from non-radioactive carrier nucleotides. The 3',5'-bisphosphates (red circles) were identified by co-migration with radiolabeled standards prepared by 5'-phosphorylation of nucleoside 3'-monophosphates with T4 polynucleotide kinase and γ - ^{32}P -ATP.

Figure 5: Synthetic 21 and 22 nt RNAs Mediate Target RNA Cleavage.

(A) Graphic representation of control 52 bp dsRNA and synthetic 21 and 22 nt dsRNAs. The sense strand of 21 and 22 nt short interfering RNAs (siRNAs) is shown blue, the antisense strand in red. The sequences of the siRNAs were derived from the cloned fragments of 52 and 111 bp dsRNAs (Figure 4A), except for the 22 nt antisense strand of duplex 5. The siRNAs in duplex 6 and 7 were unique to the 111 bp dsRNA processing reaction. The two 3' overhanging nucleotides indicated in green are present in the sequence of the synthetic antisense strand of duplexes 1 and 3. Both strands of the control 52 bp dsRNA were prepared by in vitro transcription and a fraction of transcripts may contain untemplated 3' nucleotide addition. The target RNA cleavage sites directed by the siRNA duplexes are indicated as orange circles (see legend to Figure 4A) and were determined as shown in Figure 5B. (B) Position of the cleavage sites on sense and antisense target RNAs. The target RNA sequences are as described in Figure 3B. Control 52 bp dsRNA (10 nM) or 21 and 22 nt RNA duplexes 1-7 (100 nM) were incubated with target RNA for 2.5 h at 25°C in *Drosophila* lysate. The stable 5' cleavage products were resolved on the gel. The cleavage sites are indicated in Figure 5A. The region targeted by the 52 bp

- 17 -

dsRNA or the sense (s) or antisense (as) strands are indicated by the black bars to the side of the gel. The cleavage sites are all located within the region of identity of the dsRNAs. For precise determination of the cleavage sites of the antisense strand, a lower percentage gel was used.

5

Figure 6: Long 3' overhangs on short dsRNAs inhibit RNAi.

(A) Graphic representation of 52 bp dsRNA constructs. The 3' extensions of sense and antisense strand are indicated in blue and red, respectively. The observed cleavage sites on the target RNAs are represented as orange circles analogous to Figure 4A and were determined as shown in Figure 6B. (B) Position of the cleavage sites on sense and antisense target RNAs. The target RNA sequences are as described in Figure 3B. DsRNA (10 nM) was incubated with target RNA for 2.5 h at 25°C in *Drosophila* lysate. The stable 5' cleavage products were resolved on the gel. The major cleavage sites are indicated with a horizontal arrow and also represented in Figure 6A. The region targeted by the 52 bp dsRNA is represented as black bar at both sides of the gel.

10
15

Figure 7: Proposed Model for RNAi.

RNAi is predicted to begin with processing of dsRNA (sense strand in black, antisense strand in red) to predominantly 21 and 22 nt short interfering RNAs (siRNAs). Short overhanging 3' nucleotides, if present on the dsRNA, may be beneficial for processing of short dsRNAs. The dsRNA-processing proteins, which remain to be characterized, are represented as green and blue ovals, and assembled on the dsRNA in asymmetric fashion. In our model, this is illustrated by binding of a hypothetical blue protein or protein domain with the siRNA strand in 3' to 5' direction while the hypothetical green protein or protein domain is always bound to the opposing siRNA strand. These proteins or a subset remain associated with the siRNA duplex and preserve its orientation as determined by the direction of the dsRNA processing reaction. Only the siRNA sequence associated with the blue protein is able to guide target RNA cleavage. The endonuclease com-

20
25
30

- 18 -

plex is referred to as small interfering ribonucleoprotein complex or siRNP. It is presumed here, that the endonuclease that cleaves the dsRNA may also cleave the target RNA, probably by temporarily displacing the passive siRNA strand not used for target recognition. The target RNA is then
 5 cleaved in the center of the region recognized by the sequence-complementary guide siRNA.

Figure 8: Reporter constructs and siRNA duplexes.

(a) The firefly (Pp-luc) and sea pansy (Rr-luc) luciferase reporter gene regions from plasmids pGL2-Control, pGL3-Control and pRL-TK (Promega)
 10 are illustrated. SV40 regulatory elements, the HSV thymidine kinase promoter and two introns (lines) are indicated. The sequence of GL3 luciferase is 95% identical to GL2, but RL is completely unrelated to both. Luciferase expression from pGL2 is approx. 10-fold lower than from pGL3 in trans-
 15 fected mammalian cells. The region targeted by the siRNA duplexes is indicated as black bar below the coding region of the luciferase genes. (b) The sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting GL2, GL3 and RL luciferase are shown. The GL2 and GL3 siRNA duplexes differ by only 3 single nucleotide substitutions (boxed in gray). As
 20 unspecific control, a duplex with the inverted GL2 sequence, invGL2, was synthesized. The 2 nt 3' overhang of 2'-deoxythymidine is indicated as TT; uGL2 is similar to GL2 siRNA but contains ribo-uridine 3' overhangs.

Figure 9: RNA interference by siRNA duplexes.

25 Ratios of target control luciferase were normalized to a buffer control (bu, black bars); gray bars indicate ratios of *Photinus pyralis* (Pp-luc) GL2 or GL3 luciferase to *Renilla reniformis* (Rr-luc) RL luciferase (left axis), white bars indicate RL to GL2 or GL3 ratios (right axis). Panels a, c, e, g and i describe experiments performed with the combination of pGL2-Control and
 30 pRL-TK reporter plasmids, panels b, d, f, h and j with pGL3-Control and pRL-TK reporter plasmids. The cell line used for the interference experiment is indicated at the top of each plot. The ratios of Pp-luc/Rr-luc for the

- 19 -

buffer control (bu) varied between 0.5 and 10 for pGL2/pRL and between 0.03 and 1 for pGL3/pRL, respectively, before normalization and between the various cell lines tested. The plotted data were averaged from three independent experiments \pm S.D.

5

Figure 10: Effects of 21 nt siRNA, 50 bp and 500 bp dsRNAs on luciferase expression in HeLa cells.

The exact length of the long dsRNAs is indicated below the bars. Panels a, c and e describe experiments performed with pGL2-Control and pRL-TK reporter plasmids, panels b, d and f with pGL3-Control and pRL-TK reporter plasmids. The data were averaged from two independent experiments \pm S.D. (a), (b) Absolute Pp-luc expression, plotted in arbitrary luminescence units. (c), (d) Rr-luc expression, plotted in arbitrary luminescence units. (e), (f) Ratios of normalized target to control luciferase. The ratios of luciferase activity for siRNA duplexes were normalized to a buffer control (bu, black bars); the luminescence ratios for 50 or 500 bp dsRNAs were normalized to the respective ratios observed for 50 and 500 bp dsRNA from humanized GFP (hG, black bars). It should be noted that the overall differences in sequences between the 49 and 484 bp dsRNAs targeting GL2 and GL3 are not sufficient to confer specificity between GL2 and GL3 targets (43 nt uninterrupted identity in 49 bp segment, 239 nt longest uninterrupted identity in 484 bp segment).

10

15

20

Figure 11: Variation of the 3' overhang of duplexes of 21-nt siRNAs.

(A) Outline of the experimental strategy. The capped and polyadenylated sense target mRNA is depicted and the relative positions of sense and antisense siRNAs are shown. Eight series of duplexes, according to the eight different antisense strands were prepared. The siRNA sequences and the number of overhanging nucleotides were changed in 1-nt steps. (B) Normalized relative luminescence of target luciferase (*Photinus pyralis*, Pp-luc) to control luciferase (*Renilla reniformis*, Rr-luc) in *D. melanogaster* embryo lysate in the presence of 5 nM blunt-ended dsRNAs. The lumi-

25

30

- 20 -

nescence ratios determined in the presence of dsRNA were normalized to the ratio obtained for a buffer control (bu, black bar). Normalized ratios less than 1 indicate specific interference. (C-J) Normalized interference ratios for eight series of 21-nt siRNA duplexes. The sequences of siRNA duplexes are depicted above the bar graphs. Each panel shows the interference ratio for a set of duplexes formed with a given antisense guide siRNA and 5 different sense siRNAs. The number of overhanging nucleotides (3' overhang, positive numbers; 5' overhangs, negative numbers) is indicated on the x-axis. Data points were averaged from at least 3 independent experiments, error bars represent standard deviations.

Figure 12: Variation of the length of the sense strand of siRNA duplexes. (A) Graphic representation of the experiment. Three 21-nt antisense strands were paired with eight sense siRNAs. The siRNAs were changed in length at their 3' end. The 3' overhang of the antisense siRNA was 1-nt (B), 2-nt (C), or 3-nt (D) while the sense siRNA overhang was varied for each series. The sequences of the siRNA duplexes and the corresponding interference ratios are indicated.

Figure 13: Variation of the length of siRNA duplexes with preserved 2-nt 3' overhangs.

(A) Graphic representation of the experiment. The 21-nt siRNA duplex is identical in sequence to the one shown in Figure 11H or 12C. The siRNA duplexes were extended to the 3' side of the sense siRNA (B) or the 5' side of the sense siRNA (C). The siRNA duplex sequences and the respective interference ratios are indicated.

Figure 14: Substitution of the 2'-hydroxyl groups of the siRNA ribose residues.

The 2'-hydroxyl groups (OH) in the strands of siRNA duplexes were replaced by 2'-deoxy (d) or 2'-O-methyl (Me). 2-nt and 4-nt 2'-deoxy substitu-

- 21 -

tions at the 3'-ends are indicated as 2-nt d and 4-nt d, respectively. Uridine residues were replaced by 2'-deoxy thymidine.

Figure 15: Mapping of sense and antisense target RNA cleavage by 21-nt siRNA duplexes with 2-nt 3' overhangs.

(A) Graphic representation of ³²P (asterisk) cap-labelled sense and antisense target RNAs and siRNA duplexes. The position of sense and antisense target RNA cleavage is indicated by triangles on top and below the siRNA duplexes, respectively. (B) Mapping of target RNA cleavage sites. After 2 h incubation of 10 nM target with 100 nM siRNA duplex in *D. melanogaster* embryo lysate, the 5' cap-labelled substrate and the 5' cleavage products were resolved on sequencing gels. Length markers were generated by partial RNase T1 digestion (T1) and partial alkaline hydrolysis (OH-) of the target RNAs. The bold lines to the left of the images indicate the region covered by the siRNA strands 1 and 5 of the same orientation as the target.

Figure 16: The 5' end of a guide siRNA defines the position of target RNA cleavage.

(A, B) Graphic representation of the experimental strategy. The antisense siRNA was the same in all siRNA duplexes, but the sense strand was varied between 18 to 25 nt by changing the 3' end (A) or 18 to 23 nt by changing the 5' end (B). The position of sense and antisense target RNA cleavage is indicated by triangles on top and below the siRNA duplexes, respectively. (C, D) Analysis of target RNA cleavage using cap-labelled sense (top panel) or antisense (bottom panel) target RNAs. Only the cap-labelled 5' cleavage products are shown. The sequences of the siRNA duplexes are indicated, and the length of the sense siRNA strands is marked on top of the panel. The control lane marked with a dash in panel (C) shows target RNA incubated in absence of siRNAs. Markers were as described in Figure 15. The arrows in (D), bottom panel, indicate the target RNA cleavage sites that differ by 1 nt.

Figure 17: Sequence variation of the 3' overhang of siRNA duplexes.

The 2-nt 3' overhang (NN, in gray) was changed in sequence and composition as indicated (T, 2'-deoxythymidine, dG, 2'-deoxyguanosine; asterisk, wild-type siRNA duplex). Normalized interference ratios were determined as described in Figure 11. The wild-type sequence is the same as depicted in Figure 14.

Figure 18: Sequence specificity of target recognition.

The sequences of the mismatched siRNA duplexes are shown, modified sequence segments or single nucleotides are underlayed in gray. The reference duplex (ref) and the siRNA duplexes 1 to 7 contain 2'-deoxythymidine 2-nt overhangs. The silencing efficiency of the thymidine-modified reference duplex was comparable to the wild-type sequence (Figure 17). Normalized interference ratios were determined as described in Figure 11.

Figure 19: Variation of the length of siRNA duplexes with preserved 2-nt 3' overhangs.

The siRNA duplexes were extended to the 3' side of the sense siRNA (A) or the 5' side of the sense siRNA (B). The siRNA duplex sequences and the respective interference ratios are indicated. For HeLa SS6 cells, siRNA duplexes (0.84 μ g) targeting GL2 luciferase were transfected together with pGL2-Control and pRL-TK plasmids. For comparison, the in vitro RNAi activities of siRNA duplexes tested in *D. melanogaster* lysate are indicated.

Example 1

RNA Interference Mediated by Small Synthetic RNAs

1.1. Experimental Procedures

1.1.1 In Vitro RNAi

In vitro RNAi and lysate preparations were performed as described previously (Tuschl et al., 1999; Zamore et al., 2000). It is critical to use

- 23 -

freshly dissolved creatine kinase (Roche) for optimal ATP regeneration. The RNAi translation assays (Fig. 1) were performed with dsRNA concentrations of 5 nM and an extended pre-incubation period of 15 min at 25°C prior to the addition of in vitro transcribed, capped and polyadenylated Pp-luc and Rr-luc reporter mRNAs. The incubation was continued for 1 h and the relative amount of Pp-luc and Rr-luc protein was analyzed using the dual luciferase assay (Promega) and a Monolight 3010C luminometer (PharMingen).

10 1.1.2 RNA Synthesis

Standard procedures were used for in vitro transcription of RNA from PCR templates carrying T7 or SP6 promoter sequences, see for example (Tuschl et al., 1998). Synthetic RNA was prepared using Expedite RNA phosphoramidites (Proligo). The 3' adapter oligonucleotide was synthesized using dimethoxytrityl-1,4-benzenedimethanol-succinyl-aminopropyl-CPG. The oligoribonucleotides were deprotected in 3 ml of 32% ammonia/ethanol (3/1) for 4 h at 55°C (Expedite RNA) or 16 h at 55°C (3' and 5' adapter DNA/RNA chimeric oligonucleotides) and then desilylated and gel-purified as described previously (Tuschl et al., 1993). RNA transcripts for dsRNA preparation including long 3' overhangs were generated from PCR templates that contained a T7 promoter in sense and an SP6 promoter in antisense direction. The transcription template for sense and antisense target RNA was PCR-amplified with GCGTAATACGACTCACTATAGAACAAATTGCTTTTACAG (underlined, T7 promoter) as 5' primer and ATTTAGGTGACACTATAGGCATAAAGAATTGAAGA (underlined, SP6 promoter) as 3' primer and the linearized Pp-luc plasmid (pGEM-luc sequence) (Tuschl et al., 1999) as template; the T7-transcribed sense RNA was 177 nt long with the Pp-luc sequence between pos. 113-273 relative to the start codon and followed by 17 nt of the complement of the SP6 promoter sequence at the 3' end. Transcripts for blunt-ended dsRNA

- 24 -

formation were prepared by transcription from two different PCR products which only contained a single promoter sequence.

DsRNA annealing was carried out using a phenol/chloroform extraction. Equimolar concentration of sense and antisense RNA (50 nM to 10 μ M, depending on the length and amount available) in 0.3 M NaOAc (pH 6) were incubated for 30 s at 90°C and then extracted at room temperature with an equal volume of phenol/chloroform, and followed by a chloroform extraction to remove residual phenol. The resulting dsRNA was precipitated by addition of 2.5-3 volumes of ethanol. The pellet was dissolved in lysis buffer (100 mM KCl, 30 mM HEPES-KOH, pH 7.4, 2 mM Mg(OAc)₂) and the quality of the dsRNA was verified by standard agarose gel electrophoreses in 1 x TAE-buffer. The 52 bp dsRNAs with the 17 nt and 20 nt 3' overhangs (Figure 6) were annealed by incubating for 1 min at 95 °C, then rapidly cooled to 70°C and followed by slow cooling to room temperature over a 3 h period (50 μ l annealing reaction, 1 μ M strand concentration, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5). The dsRNAs were then phenol/chloroform extracted, ethanol-precipitated and dissolved in lysis buffer.

Transcription of internally ³²P-radiolabeled RNA used for dsRNA preparation (Figures 2 and 4) was performed using 1 mM ATP, CTP, GTP, 0.1 or 0.2 mM UTP, and 0.2-0.3 μ M -³²P-UTP (3000 Ci/mmol), or the respective ratio for radiolabeled nucleoside triphosphates other than UTP. Labeling of the cap of the target RNAs was performed as described previously. The target RNAs were gel-purified after cap-labeling.

1.1.3 Cleavage Site Mapping

Standard RNAi reactions were performed by pre-incubating 10 nM dsRNA for 15 min followed by addition of 10 nM cap-labeled target RNA. The reaction was stopped after a further 2 h (Figure 2A) or 2.5 h incubation (Figure 5B and 6B) by proteinase K treatment (Tuschl et al., 1999). The samples were then analyzed on 8 or 10% sequencing gels. The 21 and 22

- 25 -

nt synthetic RNA duplexes were used at 100 nM final concentration (Fig 5B).

1.1.4 Cloning of ~21 nt RNAs

5 The 21 nt RNAs were produced by incubation of radiolabeled dsRNA in *Drosophila* lysate in absence of target RNA (200 μ l reaction, 1 h incubation, 50 nM dsP111, or 100 nM dsP52 or dsP39). The reaction mixture was subsequently treated with proteinase K (Tuschl et al., 1999) and the dsRNA-processing products were separated on a denaturing 15% poly-
10 acrylamide gel. A band, including a size range of at least 18 to 24 nt, was excised, eluted into 0.3 M NaCl overnight at 4°C and in siliconized tubes. The RNA was recovered by ethanol-precipitation and dephosphorylated (30 μ l reaction, 30 min, 50°C, 10 U alkaline phosphatase, Roche). The reaction was stopped by phenol/chloroform extraction and the RNA was ethanol-
15 precipitated. The 3' adapter oligonucleotide (pUUUaaccgcacacctctcx: uppercase, RNA; lowercase, DNA; p, phosphate; x, 4-hydroxymethylbenzyl) was then ligated to the dephosphorylated ~21 nt RNA (20 μ l reaction, 30 min, 37°C, 5 μ M 3' adapter, 50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 0.2 mM ATP, 0.1 mg/ml acetylated BSA, 15% DMSO, 25 U T4 RNA ligase, Amers-
20 ham-Pharmacia) (Pan and Uhlenbeck, 1992). The ligation reaction was stopped by the addition of an equal volume of 8 M urea/50 mM EDTA stopmix and directly loaded on a 15% gel. Ligation yields were greater 50%. The ligation product was recovered from the gel and 5'-phosphorylated (20 μ l reaction, 30 min, 37°C, 2 mM ATP, 5 U T4 polynucleotide
25 kinase, NEB). The phosphorylation reaction was stopped by phenol/chloroform extraction and RNA was recovered by ethanol-precipitation. Next, the 5' adapter (tactaatagcactcactAAA: uppercase, RNA; lowercase, DNA) was ligated to the phosphorylated ligation product as described above. The new ligation product was gel-purified and eluted from the gel slice in the
30 presence of reverse transcription primer (GACTAGCTGGAATTCAAGGATGCGGTTAAA: bold, Eco RI site) used as carrier. Reverse transcription (15 μ l reaction, 30 min, 42°C, 150 U Super-

- 26 -

script II reverse transcriptase, Life Technologies) was followed by PCR using as 5' primer CAGCCAACGGAATTCATACGACTCACTAAA (bold, Eco RI site) and the 3' RT primer. The PCR product was purified by phenol/chloroform extraction and ethanol-precipitated. The PCR product was then
5 digested with Eco RI (NEB) and concatamerized using T4 DNA ligase (high conc., NEB). Concatamers of a size range of 200 to 800 bp were separated on a low-melt agarose gel, recovered from the gel by a standard melting and phenol extraction procedure, and ethanol-precipitated. The unpaired ends were filled in by incubation with Taq polymerase under
10 standard conditions for 15 min at 72°C and the DNA product was directly ligated into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). Colonies were screened using PCR and M13-20 and M13 Reverse sequencing primers. PCR products were directly submitted for custom sequencing (Sequence Laboratories Göttingen GmbH, Germany). On average,
15 age, four to five 21mer sequences were obtained per clone.

1.1.5 2D-TLC Analysis

Nuclease P1 digestion of radiolabeled, gel-purified siRNAs and 2D-TLC was carried out as described (Zamore et al., 2000). Nuclease T2 digestion was
20 performed in 10 µl reactions for 3 h at 50°C in 10 mM ammonium acetate (pH 4.5) using 2 µg/µl carrier tRNA and 30 U ribonuclease T2 (Life Technologies). The migration of non-radioactive standards was determined by UV shadowing. The identity of nucleoside-3',5'-disphosphates was confirmed by co-migration of the T2 digestion products with standards prepared by
25 5'-³²P-phosphorylation of commercial nucleoside 3'-monophosphates using γ-³²P-ATP and T4 polynucleotide kinase (data not shown).

30

1.2 Results and Discussion

1.2.1 Length Requirements for Processing of dsRNA to 21 and 22 nt RNA Fragments

5 Lysate prepared from *D. melanogaster* syncytial embryos recapitulates RNAi in vitro providing a novel tool for biochemical analysis of the mechanism of RNAi (Tuschl et al., 1999; Zamore et al., 2000). In vitro and in vivo analysis of the length requirements of dsRNA for RNAi has revealed that short dsRNA (<150 bp) are less effective than longer dsRNAs in
10 degrading target mRNA (Caplen et al., 2000; Hammond et al., 2000; Ngo et al., 1998); Tuschl et al., 1999). The reasons for reduction in mRNA degrading efficiency are not understood. We therefore examined the precise length requirement of dsRNA for target RNA degradation under optimized conditions in the *Drosophila* lysate (Zamore et al., 2000). Several
15 series of dsRNAs were synthesized and directed against firefly luciferase (Pp-luc) reporter RNA. The specific suppression of target RNA expression was monitored by the dual luciferase assay (Tuschl et al., 1999) (Figures 1A and 1B). We detected specific inhibition of target RNA expression for dsRNAs as short as 38 bp, but dsRNAs of 29 to 36 bp were not effective
20 in this process. The effect was independent of the target position and the degree of inhibition of Pp-luc mRNA expression correlated with the length of the dsRNA, i.e. long dsRNAs were more effective than short dsRNAs.

It has been suggested that the 21-23 nt RNA fragments generated by
25 processing of dsRNAs are the mediators of RNA interference and co-suppression (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). We therefore analyzed the rate of 21-23 nt fragment formation for a subset of dsRNAs ranging in size between 501 to 29 bp. Formation of 21-23 nt fragments in *Drosophila* lysate (Figure 2) was readily
30 detectable for 39 to 501 bp long dsRNAs but was significantly delayed for the 29 bp dsRNA. This observation is consistent with a role of 21-23 nt fragments in guiding mRNA cleavage and provides an explanation for the

lack of RNAi by 30 bp dsRNAs. The length dependence of 21-23 mer formation is likely to reflect a biologically relevant control mechanism to prevent the undesired activation of RNAi by short intramolecular base-paired structures of regular cellular RNAs.

5

1.2.2 39 bp dsRNA Mediates Target RNA Cleavage at a Single Site

Addition of dsRNA and 5'-capped target RNA to the *Drosophila* lysate results in sequence-specific degradation of the target RNA (Tuschl et al., 1999). The target mRNA is only cleaved within the region of identity with the dsRNA and many of the target cleavage sites were separated by 21-23 nt (Zamore et al., 2000). Thus, the number of cleavage sites for a given dsRNA was expected to roughly correspond to the length of the dsRNA divided by 21. We mapped the target cleavage sites on a sense and an antisense target RNA which was 5' radiolabeled at the cap (Zamore et al., 2000) (Figures 3A and 3B). Stable 5' cleavage products were separated on a sequencing gel and the position of cleavage was determined by comparison with a partial RNase T1 and an alkaline hydrolysis ladder from the target RNA.

Consistent with the previous observation (Zamore et al., 2000), all target RNA cleavage sites were located within the region of identity to the dsRNA. The sense or the antisense target was only cleaved once by 39 bp dsRNA. Each cleavage site was located 10 nt from the 5' end of the region covered by the dsRNA (Figure 3B). The 52 bp dsRNA, which shares the same 5' end with the 39 bp dsRNA, produces the same cleavage site on the sense target, located 10 nt from the 5' end of the region of identity with the dsRNA, in addition to two weaker cleavage sites 23 and 24 nt downstream of the first site. The antisense target was only cleaved once, again 10 nt from the 5' end of the region covered by its respective dsRNA. Mapping of the cleavage sites for the 38 to 49 bp dsRNAs shown in Figure 1 showed that the first and predominant cleavage site was always located 7 to 10 nt downstream of the region covered by the dsRNA (data not

- 29 -

shown). This suggests that the point of target RNA cleavage is determined by the end of the dsRNA and could imply that processing to 21-23 mers starts from the ends of the duplex.

5 Cleavage sites on sense and antisense target for the longer 111 bp dsRNA were much more frequent than anticipated and most of them appear in clusters separated by 20 to 23 nt (Figures 3A and 3B). As for the shorter dsRNAs, the first cleavage site on the sense target is 10 nt from the 5' end of the region spanned by the dsRNA; and the first cleavage site on the
10 antisense target is located 9 nt from the 5' end of region covered by the dsRNA. It is unclear what causes this disordered cleavage, but one possibility could be that longer dsRNAs may not only get processed from the ends but also internally, or there are some specificity determinants for dsRNA processing which we do not yet understand. Some irregularities to
15 the 21-23 nt spacing were also previously noted (Zamore et al., 2000). To better understand the molecular basis of dsRNA processing and target RNA recognition, we decided to analyze the sequences of the 21-23 nt fragments generated by processing of 39, 52, and 111 bp dsRNAs in the *Drosophila* lysate.

20

1.2.3 dsRNA is Processed to 21 and 22 nt RNAs by an RNase III-Like Mechanism

In order to characterize the 21-23 nt RNA fragments we examined the 5' and 3' termini of the RNA fragments. Periodate oxidation of gel-purified 21-
25 23 nt RNAs followed by β -elimination indicated the presence of a terminal 2' and 3' hydroxyl groups. The 21-23 mers were also responsive to alkaline phosphatase treatment indicating the presence of a 5' terminal phosphate group. The presence of 5' phosphate and 3' hydroxyl termini suggests that the dsRNA could be processed by an enzymatic activity
30 similar to *E. coli* RNase III (for reviews, see (Dunn, 1982; Nicholson, 1999; Robertson, 1990; Robertson, 1982)).

- 30 -

Directional cloning of 21-23 nt RNA fragments was performed by ligation of a 3' and 5' adapter oligonucleotide to the purified 21-23 mers using T4 RNA ligase. The ligation products were reverse transcribed, PCR-amplified, concatamerized, cloned, and sequenced. Over 220 short RNAs were
5 sequenced from dsRNA processing reactions of the 39, 52 and 111 bp dsRNAs (Figure 4A). We found the following length distribution: 1% 18 nt, 5% 19 nt, 12% 20 nt, 45% 21 nt, 28% 22 nt, 6% 23 nt, and 2% 24 nt. Sequence analysis of the 5' terminal nucleotide of the processed fragments indicated that oligonucleotides with a 5' guanosine were underrepresented.
10 This bias was most likely introduced by T4 RNA ligase which discriminates against 5' phosphorylated guanosine as donor oligonucleotide; no significant sequence bias was seen at the 3' end. Many of the ~21 nt fragments derived from the 3' ends of the sense or antisense strand of the duplexes include 3' nucleotides that are derived from untemplated addition of nu-
15 cleotides during RNA synthesis using T7 RNA polymerase. Interestingly, a significant number of endogenous *Drosophila* ~21 nt RNAs were also cloned, some of them from LTR and non-LTR retrotransposons (data not shown). This is consistent with a possible role for RNAi in transposon silencing.
20
The ~21 nt RNAs appear in clustered groups (Figure 4A) which cover the entire dsRNA sequences. Apparently, the processing reaction cuts the dsRNA by leaving staggered 3' ends, another characteristic of RNase III cleavage. For the 39 bp dsRNA, two clusters of ~21 nt RNAs were found
25 from each dsRNA-constituting strand including overhanging 3' ends, yet only one cleavage site was detected on the sense and antisense target (Figures 3A and 3B). If the ~21 nt fragments were present as single-stranded guide RNAs in a complex that mediates mRNA degradation, it could be assumed that at least two target cleavage sites exist, but this
30 was not the case. This suggests that the ~21 nt RNAs may be present in double-stranded form in the endonuclease complex but that only one of the strands can be used for target RNA recognition and cleavage. The use of

- 31 -

only one of the ~21 nt strands for target cleavage may simply be determined by the orientation in which the ~21 nt duplex is bound to the nuclease complex. This orientation is defined by the direction in which the original dsRNA was processed.

5

The ~21mer clusters for the 52 bp and 111 bp dsRNA are less well defined when compared to the 39 bp dsRNA. The clusters are spread over regions of 25 to 30 nt most likely representing several distinct subpopulations of ~21 nt duplexes and therefore guiding target cleavage at several nearby sites. These cleavage regions are still predominantly separated by 20 to 23 nt intervals. The rules determining how regular dsRNA can be processed to ~21 nt fragments are not yet understood, but it was previously observed that the approx. 21-23 nt spacing of cleavage sites could be altered by a run of uridines (Zamore et al., 2000). The specificity of dsRNA cleavage by *E. coli* RNase III appears to be mainly controlled by antideterminants, i.e. excluding some specific base-pairs at given positions relative to the cleavage site (Zhang and Nicholson, 1997).

To test whether sugar-, base- or cap-modification were present in processed ~21 nt RNA fragments, we incubated radiolabeled 505 bp Pp-luc dsRNA in lysate for 1 h, isolated the ~21 nt products, and digested it with P1 or T2 nuclease to mononucleotides. The nucleotide mixture was then analyzed by 2D thin-layer chromatography (Figure 4B). None of the four natural ribonucleotides were modified as indicated by P1 or T2 digestion. We have previously analyzed adenosine to inosine conversion in the ~21 nt fragments (after a 2 h incubation) and detected a small extent (<0.7%) deamination (Zamore et al., 2000); shorter incubation in lysate (1 h) reduced this inosine fraction to barely detectable levels. RNase T2, which cleaves 3' of the phosphodiester linkage, produced nucleoside 3'-phosphate and nucleoside 3',5'-diphosphate, thereby indicating the presence of a 5'-terminal monophosphate. All four nucleoside 3',5'-diphosphates were detected and suggest that the internucleotidic linkage was

20

25

30

- 32 -

cleaved with little or no sequence-specificity. In summary, the ~21 nt fragments are unmodified and were generated from dsRNA such that 5'-monophosphates and 3'-hydroxyls were present at the 5'-end.

5 **1.2.4 Synthetic 21 and 22 nt RNAs Mediate Target RNA Cleavage**

Analysis of the products of dsRNA processing indicated that the ~21 nt fragments are generated by a reaction with all the characteristics of an RNase III cleavage reaction (Dunn, 1982; Nicholson, 1999; Robertson, 1990; Robertson, 1982). RNase III makes two staggered cuts in both
10 strands of the dsRNA, leaving a 3' overhang of about 2 nt. We chemically synthesized 21 and 22 nt RNAs, identical in sequence to some of the cloned ~21 nt fragments, and tested them for their ability to mediate target RNA degradation (Figures 5A and 5B). The 21 and 22 nt RNA duplexes were incubated at 100 nM concentrations in the lysate, a 10-fold
15 higher concentrations than the 52 bp control dsRNA. Under these conditions, target RNA cleavage is readily detectable. Reducing the concentration of 21 and 22 nt duplexes from 100 to 10 nM does still cause target RNA cleavage. Increasing the duplex concentration from 100 nM to 1000 nM however does not further increase target cleavage, probably due to a
20 limiting protein factor within the lysate.

In contrast to 29 or 30 bp dsRNAs that did not mediate RNAi, the 21 and 22 nt dsRNAs with overhanging 3' ends of 2 to 4 nt mediated efficient degradation of target RNA (duplexes 1, 3, 4, 6, Figures 5A and 5B). Blunt-
25 ended 21 or 22 nt dsRNAs (duplexes 2, 5, and 7, Figures 5A and 5B) were reduced in their ability to degrade the target and indicate that overhanging 3' ends are critical for reconstitution of the RNA-protein nuclease complex. The single-stranded overhangs may be required for high affinity binding of the ~21 nt duplex to the protein components. A 5' terminal phosphate,
30 although present after dsRNA processing, was not required to mediate target RNA cleavage and was absent from the short synthetic RNAs.

- 33 -

The synthetic 21 and 22 nt duplexes guided cleavage of sense as well as antisense targets within the region covered by the short duplex. This is an important result considering that a 39 bp dsRNA, which forms two pairs of clusters of ~21 nt fragments (Fig. 2), cleaved sense or antisense target only once and not twice. We interpret this result by suggesting that only one of two strands present in the ~21 nt duplex is able to guide target RNA cleavage and that the orientation of the ~21 nt duplex in the nuclease complex is determined by the initial direction of dsRNA processing. The presentation of an already perfectly processed ~21 nt duplex to the in vitro system however does allow formation of the active sequence-specific nuclease complex with two possible orientations of the symmetric RNA duplex. This results in cleavage of sense as well as antisense target within the region of identity with the 21 nt RNA duplex.

The target cleavage site is located 11 or 12 nt downstream of the first nucleotide that is complementary to the 21 or 22 nt guide sequence, i.e. the cleavage site is near center of the region covered by the 21 or 22 nt RNAs (Figures 4A and 4B). Displacing the sense strand of a 22 nt duplex by two nucleotides (compare duplexes 1 and 3 in Figure 5A) displaced the cleavage site of only the antisense target by two nucleotides. Displacing both sense and antisense strand by two nucleotides shifted both cleavage sites by two nucleotides (compare duplexes 1 and 4). We predict that it will be possible to design a pair of 21 or 22 nt RNAs to cleave a target RNA at almost any given position.

The specificity of target RNA cleavage guided by 21 and 22 nt RNAs appears exquisite as no aberrant cleavage sites are detected (Figure 5B). It should however be noted, that the nucleotides present in the 3' overhang of the 21 and 22 nt RNA duplex may contribute less to substrate recognition than the nucleotides near the cleavage site. This is based on the observation that the 3' most nucleotide in the 3' overhang of the active duplexes 1 or 3 (Figure 5A) is not complementary to the target. A detailed

- 34 -

analysis of the specificity of RNAi can now be readily undertaken using synthetic 21 and 22 nt RNAs.

5 Based on the evidence that synthetic 21 and 22 nt RNAs with overhanging 3' ends mediate RNA interference, we propose to name the ~21 nt RNAs "short interfering RNAs" or siRNAs and the respective RNA-protein complex a "small interfering ribonucleoprotein particle" or siRNP.

1.2.5 3' Overhangs of 20 nt on short dsRNAs inhibit RNAi

10 We have shown that short blunt-ended dsRNAs appear to be processed from the ends of the dsRNA. During our study of the length dependence of dsRNA in RNAi, we have also analyzed dsRNAs with 17 to 20 nt overhanging 3' ends and found to our surprise that they were less potent than blunt-ended dsRNAs. The inhibitory effect of long 3' ends was particularly pronounced for dsRNAs up to 100 bp but was less dramatic for longer dsRNAs. The effect was not due to imperfect dsRNA formation based on native gel analysis (data not shown). We tested if the inhibitory effect of long overhanging 3' ends could be used as a tool to direct dsRNA processing to only one of the two ends of a short RNA duplex.

20

We synthesized four combinations of the 52 bp model dsRNA, blunt-ended, 3' extension on only the sense strand, 3'-extension on only the antisense strand, and double 3' extension on both strands, and mapped the target RNA cleavage sites after incubation in lysate (Figures 6A and 25 6B). The first and predominant cleavage site of the sense target was lost when the 3' end of the antisense strand of the duplex was extended, and vice versa, the strong cleavage site of the antisense target was lost when the 3' end of sense strand of the duplex was extended. 3' Extensions on both strands rendered the 52 bp dsRNA virtually inactive. One explanation 30 for the dsRNA inactivation by ~20 nt 3' extensions could be the association of single-stranded RNA-binding proteins which could interfere with the association of one of the dsRNA-processing factors at this end. This result

- 35 -

is also consistent with our model where only one of the strands of the siRNA duplex in the assembled siRNP is able to guide target RNA cleavage. The orientation of the strand that guides RNA cleavage is defined by the direction of the dsRNA processing reaction. It is likely that the presence of 3' staggered ends may facilitate the assembly of the processing complex. A block at the 3' end of the sense strand will only permit dsRNA processing from the opposing 3' end of the antisense strand. This in turn generates siRNP complexes in which only the antisense strand of the siRNA duplex is able to guide sense target RNA cleavage. The same is true for the reciprocal situation.

The less pronounced inhibitory effect of long 3' extensions in the case of longer dsRNAs (≥ 500 bp, data not shown) suggests to us that long dsRNAs may also contain internal dsRNA-processing signals or may get processed cooperatively due to the association of multiple cleavage factors.

1.2.6 A Model for dsRNA-Directed mRNA Cleavage

The new biochemical data update the model for how dsRNA targets mRNA for destruction (Figure 7). Double-stranded RNA is first processed to short RNA duplexes of predominantly 21 and 22 nt in length and with staggered 3' ends similar to an RNase III-like reaction (Dunn, 1982; Nicholson, 1999; Robertson, 1982). Based on the 21-23 nt length of the processed RNA fragments it has already been speculated that an RNase III-like activity may be involved in RNAi (Bass, 2000). This hypothesis is further supported by the presence of 5' phosphates and 3' hydroxyls at the termini of the siRNAs as observed in RNase III reaction products (Dunn, 1982; Nicholson, 1999). Bacterial RNase III and the eukaryotic homologs Rnt1p in *S. cerevisiae* and Pac1p in *S. pombe* have been shown to function in processing of ribosomal RNA as well as snRNA and snoRNAs (see for example Chanfreau et al., 2000).

- 36 -

Little is known about the biochemistry of RNase III homologs from plants, animals or human. Two families of RNase III enzymes have been identified predominantly by database-guided sequence analysis or cloning of cDNAs. The first RNase III family is represented by the 1327 amino acid long D. melanogaster protein drosha (Acc. AF116572). The C-terminus is composed of two RNase III and one dsRNA-binding domain and the N-terminus is of unknown function. Close homologs are also found in *C. elegans* (Acc. AF160248) and human (Acc. AF189011) (Filippov et al., 2000; Wu et al., 2000). The drosha-like human RNase III was recently cloned and characterized (Wu et al., 2000). The gene is ubiquitously expressed in human tissues and cell lines, and the protein is localized in the nucleus and the nucleolus of the cell. Based on results inferred from antisense inhibition studies, a role of this protein for rRNA processing was suggested. The second class is represented by the *C. elegans* gene K12H4.8 (Acc. S44849) coding for a 1822 amino acid long protein. This protein has an N-terminal RNA helicase motif which is followed by 2 RNase III catalytic domains and a dsRNA-binding motif, similar to the drosha RNase III family. There are close homologs in *S. pombe* (Acc. Q09884), *A. thaliana* (Acc. AF187317), *D. melanogaster* (Acc. AE003740), and human (Acc. AB028449) (Filippov et al., 2000; Jacobsen et al., 1999; Matsuda et al., 2000). Possibly the K12H4.8 RNase III/helicase is the likely candidate to be involved in RNAi.

Genetic screens in *C. elegans* identified *rde-1* and *rde-4* as essential for activation of RNAi without an effect on transposon mobilization or co-suppression (Dernburg et al., 2000; Grishok et al., 2000; Ketting and Plasterk, 2000; Tabara et al., 1999). This led to the hypothesis that these genes are important for dsRNA processing but are not involved in mRNA target degradation. The function of both genes is as yet unknown, the *rde-1* gene product is a member of a family of proteins similar to the rabbit protein eIF2C (Tabara et al., 1999), and the sequence of *rde-4* has not yet

- 37 -

been described. Future biochemical characterization of these proteins should reveal their molecular function.

Processing to the siRNA duplexes appears to start from the ends of both
5 blunt-ended dsRNAs or dsRNAs with short (1-5 nt) 3' overhangs, and
proceeds in approximately 21-23 nt steps. Long (~20 nt) 3' staggered
ends on short dsRNAs suppress RNAi, possibly through interaction with
single-stranded RNA-binding proteins. The suppression of RNAi by single-
stranded regions flanking short dsRNA and the lack of siRNA formation
10 from short 30 bp dsRNAs may explain why structured regions frequently
encountered in mRNAs do not lead to activation of RNAi.

Without wishing to be bound by theory, we presume that the dsRNA-
processing proteins or a subset of these remain associated with the siRNA
15 duplex after the processing reaction. The orientation of the siRNA duplex
relative to these proteins determines which of the two complementary
strands functions in guiding target RNA degradation. Chemically syn-
thesized siRNA duplexes guide cleavage of sense as well as antisense
target RNA as they are able to associate with the protein components in
20 either of the two possible orientation.

The remarkable finding that synthetic 21 and 22 nt siRNA duplexes can be
used for efficient mRNA degradation provides new tools for sequence-
specific regulation of gene expression in functional genomics as well as
25 biomedical studies. The siRNAs may be effective in mammalian systems
where long dsRNAs cannot be used due to the activation of the PKR
response (Clemens, 1997). As such, the siRNA duplexes represent a new
alternative to antisense or ribozyme therapeutics.

30

Example 2

RNA Interference in Human Tissue Cultures

2.1 Methods

5

2.1.1 RNA preparation

21 nt RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Germany). Synthetic oligonucleotides were deprotected and gel-purified (Example 1), followed by Sep-Pak C18 cartridge (Waters, Milford, MA, USA) purification (Tuschl, 1993). The siRNA sequences targeting GL2 (Acc. X65324) and GL3 luciferase (Acc. U47296) corresponded to the coding regions 153-173 relative to the first nucleotide of the start codon, siRNAs targeting RL (Acc. AF025846) corresponded to region 119-129 after the start codon. Longer RNAs were transcribed with T7 RNA polymerase from PCR products, followed by gel and Sep-Pak purification. The 49 and 484 bp GL2 or GL3 dsRNAs corresponded to position 113-161 and 113-596, respectively, relative to the start of translation; the 50 and 501 bp RL dsRNAs corresponded to position 118-167 and 118-618, respectively. PCR templates for dsRNA synthesis targeting humanized GFP (hG) were amplified from pAD3 (Kehlenbach, 1998), whereby 50 and 501 bp hG dsRNA corresponded to position 118-167 and 118-618, respectively, to the start codon.

15
20

For annealing of siRNAs, 20 μ M single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C followed by 1 h at 37°C. The 37°C incubation step was extended overnight for the 50 and 500 bp dsRNAs and these annealing reactions were performed at 8.4 μ M and 0.84 μ M strand concentrations, respectively.

25
30

2.1.2 Cell Culture

S2 cells were propagated in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin at 25°C. 293, NIH/3T3, HeLa S3, COS-7 cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were regularly passaged to maintain exponential growth. 24 h before transfection at approx. 80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics ($1-3 \times 10^5$ cells/ml) and transferred to 24-well plates (500 µl/well). S2 cells were not trypsinized before splitting. Transfection was carried out with Lipofectamine 2000 reagent (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 µg pGL2-Control (Promega) or pGL3-Control (Promega), 0.1 µg pRL-TK (Promega) and 0.28 µg siRNA duplex or dsRNA, formulated into liposomes, were applied; the final volume was 600 µl per well. Cells were incubated 20 h after transfection and appeared healthy thereafter. Luciferase expression was subsequently monitored with the Dual luciferase assay (Promega). Transfection efficiencies were determined by fluorescence microscopy for mammalian cell lines after co-transfection of 1.1 µg hGFP-encoding pAD3 and 0.28 µg invGL2 inGL2 siRNA and were 70-90%. Reporter plasmids were amplified in XL-1 Blue (Stratagene) and purified using the Qiagen EndoFree Maxi Plasmid Kit.

2.2 Results and Discussion

To test whether siRNAs are also capable of mediating RNAi in tissue culture, we synthesized 21 nt siRNA duplexes with symmetric 2 nt 3' overhangs directed against reporter genes coding for sea pansy (*Renilla reniformis*) and two sequence variants of firefly (*Photinus pyralis*, GL2 and GL3) luciferases (Fig. 8a, b). The siRNA duplexes were co-transfected with the reporter plasmid combinations pGL2/pRL or pGL3/pRL into *D. melanogaster* Schneider S2 cells or mammalian cells using cationic liposomes. Luciferase activities were determined 20 h after transfection. In all cell lines tested,

- 40 -

we observed specific reduction of the expression of the reporter genes in the presence of cognate siRNA duplexes (Fig. 9a-j). Remarkably, the absolute luciferase expression levels were unaffected by non-cognate siRNAs, indicating the absence of harmful side effects by 21 nt RNA duplexes (e.g. Fig. 10a-d for HeLa cells). In *D. melanogaster* S2 cells (Fig. 9a, b), the specific inhibition of luciferases was complete. In mammalian cells, where the reporter genes were 50- to 100-fold stronger expressed, the specific suppression was less complete (Fig. 9c-j). GL2 expression was reduced 3- to 12-fold, GL3 expression 9- to 25-fold and RL expression 1- to 3-fold, in response to the cognate siRNAs. For 293 cells, targeting of RL luciferase by RL siRNAs was ineffective, although GL2 and GL3 targets responded specifically (Fig. 9i, j). The lack of reduction of RL expression in 293 cells may be due to its 5- to 20-fold higher expression compared to any other mammalian cell line tested and/or to limited accessibility of the target sequence due to RNA secondary structure or associated proteins. Nevertheless, specific targeting of GL2 and GL3 luciferase by the cognate siRNA duplexes indicated that RNAi is also functioning in 293 cells.

The 2 nt 3' overhang in all siRNA duplexes, except for uGL2, was composed of (2'-deoxy) thymidine. Substitution of uridine by thymidine in the 3' overhang was well tolerated in the *D. melanogaster* in vitro system and the sequence of the overhang was uncritical for target recognition. The thymidine overhang was chosen, because it is supposed to enhance nuclease resistance of siRNAs in the tissue culture medium and within transfected cells. Indeed, the thymidine-modified GL2 siRNA was slightly more potent than the unmodified uGL2 siRNA in all cell lines tested (Fig. 9a, c, e, g, i). It is conceivable that further modifications of the 3' overhanging nucleotides may provide additional benefits to the delivery and stability of siRNA duplexes.

In co-transfection experiments, 25 nM siRNA duplexes with respect to the final volume of tissue culture medium were used (Fig. 9, 10). Increasing

- 41 -

the siRNA concentration to 100 nM did not enhance the specific silencing effects, but started to affect transfection efficiencies due to competition for liposome encapsulation between plasmid DNA and siRNA (data not shown). Decreasing the siRNA concentration to 1.5 nM did not reduce the specific silencing effect (data not shown), even though the siRNAs were now only 2- to 20-fold more concentrated than the DNA plasmids. This indicates that siRNAs are extraordinarily powerful reagents for mediating gene silencing and that siRNAs are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene targeting experiments.

In order to monitor the effect of longer dsRNAs on mammalian cells, 50 and 500 bp dsRNAs cognate to the reporter genes were prepared. As non-specific control, dsRNAs from humanized GFP (hG) (Kehlenbach, 1998) was used. When dsRNAs were co-transfected, in identical amounts (not concentrations) to the siRNA duplexes, the reporter gene expression was strongly and unspecifically reduced. This effect is illustrated for HeLa cells as a representative example (Fig. 10a-d). The absolute luciferase activities were decreased unspecifically 10- to 20-fold by 50 bp dsRNA and 20- to 200-fold by 500 bp dsRNA co-transfection, respectively. Similar unspecific effects were observed for COS-7 and NIH/3T3 cells. For 293 cells, a 10- to 20-fold unspecific reduction was observed only for 500 bp dsRNAs. Unspecific reduction in reporter gene expression by dsRNA > 30 bp was expected as part of the interferon response.

Surprisingly, despite the strong unspecific decrease in reporter gene expression, we reproducibly detected additional sequence-specific, dsRNA-mediated silencing. The specific silencing effects, however, were only apparent when the relative reporter gene activities were normalized to the hG dsRNA controls (Fig. 10e, f). A 2- to 10-fold specific reduction in response to cognate dsRNA was observed, also in the other three mammalian cell lines tested (data not shown). Specific silencing effects with

- 42 -

dsRNAs (356-1662 bp) were previously reported in CHO-K1 cells, but the amounts of dsRNA required to detect a 2- to 4-fold specific reduction were about 20-fold higher than in our experiments (Ui-Tei, 2000). Also CHO-K1 cells appear to be deficient in the interferon response. In another report, 5 293, NIH/3T3 and BHK-21 cells were tested for RNAi using luciferase/lacZ reporter combinations and 829 bp specific lacZ or 717 bp unspecific GFP dsRNA (Caplen, 2000). The failure of detecting RNAi in this case may be due to the less sensitive luciferase/lacZ reporter assay and the length differences of target and control dsRNA. Taken together, our results indicate that RNAi is active in mammalian cells, but that the silencing effect is 10 difficult to detect, if the interferon system is activated by dsRNA > 30 bp.

In summary, we have demonstrated for the first time siRNA-mediated gene silencing in mammalian cells. The use of short siRNAs holds great promise 15 for inactivation of gene function in human tissue culture and the development of gene-specific therapeutics.

Example 3

Specific Inhibition of Gene Expression by RNA Interference

20

3.1 Materials and Methods

3.1.1 RNA preparation and RNAi assay

Chemical RNA synthesis, annealing, and luciferase-based RNAi assays 25 were performed as described in Examples 1 or 2 or in previous publications (Tuschl et al., 1999; Zamore et al., 2000). All siRNA duplexes were directed against firefly luciferase, and the luciferase mRNA sequence was derived from pGEM-luc (GenBank acc. X65316) as described (Tuschl et al., 1999). The siRNA duplexes were incubated in *D. melanogaster* RNAi/trans- 30 lation reaction for 15 min prior to addition of mRNAs. Translation-based RNAi assays were performed at least in triplicates.

- 43 -

For mapping of sense target RNA cleavage, a 177-nt transcript was generated, corresponding to the firefly luciferase sequence between positions 113-273 relative to the start codon, followed by the 17-nt complement of the SP6 promoter sequence. For mapping of antisense target RNA cleavage, a 166-nt transcript was produced from a template, which was amplified from plasmid sequence by PCR using 5' primer TAATACGACTCACTATAGAGCCCATATCGTTTCATA (T7 promoter underlined) and 3' primer AGAGGATGGAACCGCTGG. The target sequence corresponds to the complement of the firefly luciferase sequence between positions 50-215 relative to the start codon. Guanylyl transferase labelling was performed as previously described (Zamore et al., 2000). For mapping of target RNA cleavage, 100 nM siRNA duplex was incubated with 5 to 10 nM target RNA in *D. melanogaster* embryo lysate under standard conditions (Zamore et al., 2000) for 2 h at 25°C. The reaction was stopped by the addition of 8 volumes of proteinase K buffer (200 mM Tris-HCl pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% w/v sodium dodecyl sulfate). Proteinase K (E.M. Merck, dissolved in water) was added to a final concentration of 0.6 mg/ml. The reactions were then incubated for 15 min at 65°C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with 3 volumes of ethanol. Samples were located on 6% sequencing gels. Length standards were generated by partial RNase T1 digestion and partial base hydrolysis of the cap-labelled sense or antisense target RNAs.

3.2 Results

3.2.1 Variation of the 3' overhang in duplexes of 21-nt siRNAs

As described above, 2 or 3 unpaired nucleotides at the 3' end of siRNA duplexes were more efficient in target RNA degradation than the respective blunt-ended duplexes. To perform a more comprehensive analysis of the function of the terminal nucleotides, we synthesized five 21-nt sense siRNAs, each displayed by one nucleotide relative to the target RNA, and eight 21-nt antisense siRNAs, each displaced by one nucleotide relative to

- 44 -

the target (Figure 11A). By combining sense and antisense siRNAs, eight series of siRNA duplexes with synthetic overhanging ends were generated covering a range of 7-nt 3' overhang to 4-nt 5' overhang. The interference of siRNA duplexes was measured using the dual luciferase assay system (Tuschl et al., 1999; Zamore et al., 2000). siRNA duplexes were directed against firefly luciferase mRNA, and sea pansy luciferase mRNA was used as internal control. The luminescence ratio of target to control luciferase activity was determined in the presence of siRNA duplex and was normalized to the ratio observed in the absence of dsRNA. For comparison, the interference ratios of long dsRNAs (39 to 504 pb) are shown in Figure 11B. The interference ratios were determined at concentrations of 5 nM for long dsRNAs (Figure 11A) and at 100 nM for siRNA duplexes (Figure 11C-J). The 100 nM concentrations of siRNAs was chosen, because complete processing of 5 nM 504 bp dsRNA would result in 120 nM total siRNA duplexes.

The ability of 21-nt siRNA duplexes to mediate RNAi is dependent on the number of overhanging nucleotides or base pairs formed. Duplexes with four to six 3' overhanging nucleotides were unable to mediate RNAi (Figure 11C-F), as were duplexes with two or more 5' overhanging nucleotides (Figure 11G-J). The duplexes with 2-nt 3' overhangs were most efficient in mediating RNA interference, though the efficiency of silencing was also sequence-dependent, and up to 12-fold differences were observed for different siRNA duplexes with 2-nt 3' overhangs (compare Figure 11D-H). Duplexes with blunted ends, 1-nt 5' overhang or 1- to 3-nt 3' overhangs were sometimes functional. The small silencing effect observed for the siRNA duplex with 7-nt 3' overhang (Figure 11C) may be due to an antisense effect of the long 3' overhang rather than due to RNAi. Comparison of the efficiency of RNAi between long dsRNAs (Fig. 11B) and the most effective 21-nt siRNA duplexes (Fig. 11E, G, H) indicates that a single siRNA duplex at 100 nM concentration can be as effective as 5 nM 504 bp dsRNA.

3.2.2 Length variation of the sense siRNA paired to an invariant 21-nt antisense siRNA

In order to investigate the effect of length of siRNA on RNAi, we prepared 3 series of siRNA duplexes, combining three 21-nt antisense strands with eight, 18- to 25-nt sense strands. The 3' overhang of the antisense siRNA was fixed to 1, 2, or 3 nt in each siRNA duplex series, while the sense siRNA was varied at its 3' end (Figure 12A). Independent of the length of the sense siRNA, we found that duplexes with 2-nt 3' overhang of antisense siRNA (Figure 12C) were more active than those with 1- or 3-nt 3' overhang (Figure 12B, D). In the first series, with 1-nt 3' overhang of antisense siRNA, duplexes with a 21- and 22-nt sense siRNAs, carrying a 1- and 2-nt 3' overhang of sense siRNA, respectively, were most active. Duplexes with 19- to 25-nt sense siRNAs were also able to mediate RNAi, but to a lesser extent. Similarly, in the second series, with 2-nt overhang of antisense siRNA, the 21-nt siRNA duplex with 2-nt 3' overhang was most active, and any other combination with the 18- to 25-nt sense siRNAs was active to a significant degree. In the last series, with 3-nt antisense siRNA 3' overhang, only the duplex with a 20-nt sense siRNA and the 2-nt sense 3' overhang was able to reduce target RNA expression. Together, these results indicate that the length of the siRNA as well as the length of the 3' overhang are important, and that duplexes of 21-nt siRNAs with 2-nt 3' overhang are optimal for RNAi.

3.2.3 Length variation of siRNA duplexes with a constant 2-nt 3' overhang

We then examined the effect of simultaneously changing the length of both siRNA strands by maintaining symmetric 2-nt 3' overhangs (Figure 13A). Two series of siRNA duplexes were prepared including the 21-nt siRNA duplex of Figure 11H as reference. The length of the duplexes was varied between 20 to 25 bp by extending the base-paired segment at the 3' end of the sense siRNA (Figure 13B) or at the 3' end of the antisense siRNA (Figure 13C). Duplexes of 20 to 23 bp caused specific repression of target luciferase activity, but the 21-nt siRNA duplex was at least 8-fold more

- 46 -

efficient than any of the other duplexes. 24- and 25-nt siRNA duplexes did not result in any detectable interference. Sequence-specific effects were minor as variations on both ends of the duplex produced similar effects.

5 **3.2.4 2'-Deoxy and 2'-O-methyl-modified siRNA duplexes**

To assess the importance of the siRNA ribose residues for RNAi, duplexes with 21-nt siRNAs and 2-nt 3' overhangs with 2'-deoxy- or 2'-O-methyl-modified strands were examined (Figure 14). Substitution of the 2-nt 3' overhangs by 2'-deoxy nucleotides had no effect, and even the replacement of two additional ribonucleotides adjacent to the overhangs in the
10 paired region, produced significantly active siRNAs. Thus, 8 out of 42 nt of a siRNA duplex were replaced by DNA residues without loss of activity. Complete substitution of one or both siRNA strands by 2'-deoxy residues, however, abolished RNAi, as did substitution by 2'-O-methyl residues.

15

3.2.5 Definition of target RNA cleavage sites

Target RNA cleavage positions were previously determined for 22-nt siRNA duplexes and for a 21-nt/22-nt duplex. It was found that the position of the target RNA cleavage was located in the centre of the region covered by
20 the siRNA duplex, 11 or 12 nt downstream of the first nucleotide that was complementary to the 21- or 22-nt siRNA guide sequence. Five distinct 21-nt siRNA duplexes with 2-nt 3' overhang (Figure 15A) were incubated with 5' cap-labelled sense or antisense target RNA in *D. melanogaster* lysate (Tuschl et al., 1999; Zamore et al., 2000). The 5' cleavage products were
25 resolved on sequencing gels (Figure 15B). The amount of sense target RNA cleaved correlates with the efficiency of siRNA duplexes determined in the translation-based assay, and siRNA duplexes 1, 2 and 4 (Figure 15B and 11H, G, E) cleave target RNA faster than duplexes 3 and 5 (Figure 15B and 11F, D). Notably, the sum of radioactivity of the 5' cleavage product and
30 the input target RNA were not constant over time, and the 5' cleavage products did not accumulate. Presumably, the cleavage products, once

- 47 -

released from the siRNA-endonuclease complex, are rapidly degraded due to the lack of either of the poly(A) tail of the 5'-cap.

5 The cleavage sites for both, sense and antisense target RNAs were located in the middle of the region spanned by the siRNA duplexes. The cleavage sites for each target produced by the 5 different duplexes varied by 1-nt according to the 1-nt displacement of the duplexes along the target sequences. The targets were cleaved precisely 11 nt downstream of the target position complementary to the 3'-most nucleotide of the sequence-
10 complementary guide siRNA (Figure 15A, B).

In order to determine, whether the 5' or the 3' end of the guide siRNA sets the ruler for target RNA cleavage, we devised the experimental strategy outlined in Figure 16A and B. A 21-nt antisense siRNA, which was kept
15 invariant for this study, was paired with sense siRNAs that were modified at either of their 5' or 3' ends. The position of sense and antisense target RNA cleavage was determined as described above. Changes in the 3' end of the sense siRNA, monitored for 1-nt 5' overhang to 6-nt 3' overhang, did neither effect the position of sense nor antisense target RNA cleavage
20 (Figure 16C). Changes in the 5' end of the sense siRNA did no affect the sense target RNA cleavage (Figure 16D, top panel), which was expected because the antisense siRNA was unchanged. However, the antisense target RNA cleavage was affected and strongly dependent on the 5' end of the sense siRNA (Figure 16D, bottom panel). The antisense target was only
25 cleaved, when the sense siRNA was 20 or 21 nt in size, and the position of cleavage different by 1-nt, suggesting that the 5' end of the target-recognizing siRNA sets the ruler for target RNA cleavage. The position is located between nucleotide 10 and 11 when counting in upstream direction from the target nucleotide paired to the 5'-most nucleotide of the
30 guide siRNA (see also Figure 15A).

- 48 -

3.2.6 Sequence effects and 2'-deoxy substitutions in the 3' overhang

A 2-nt 3' overhang is preferred for siRNA function. We wanted to know, if the sequence of the overhanging nucleotides contributes to target recognition, or if it is only a feature required for reconstitution of the endonuclease complex (RISC or siRNP). We synthesized sense and antisense siRNAs with AA, CC, GG, UU, and UG 3' overhangs and included the 2'-deoxy modifications TdG and TT. The wild-type siRNAs contained AA in the sense 3' overhang and UG in the antisense 3' overhang (AA/UG). All siRNA duplexes were functional in the interference assay and reduced target expression at least 5-fold (Figure 17). The most efficient siRNA duplexes that reduced target expression more than 10-fold, were of the sequence type NN/UG, NN/UU, NN/TdG, and NN/TT (N, any nucleotide). siRNA duplexes with an antisense siRNA 3' overhang of AA, CC or GG were less active by a factor 2 to 4 when compared to the wild-type sequence UG or the mutant UU. This reduction in RNAi efficiency is likely due to the contribution of the penultimate 3' nucleotide to sequence-specific target recognition, as the 3' terminal nucleotide was changed from G to U without effect.

Changes in the sequence of the 3' overhang of the sense siRNA did not reveal any sequence-dependent effects, which was expected, because the sense siRNA must not contribute to sense target mRNA recognition.

3.2.7 Sequence specificity of target recognition

In order to examine the sequence-specificity of target recognition, we introduced sequence changes into the paired segments of siRNA duplexes and determined the efficiency of silencing. Sequence changes were introduced by inverting short segments of 3- or 4-nt length or as point mutations (Figure 18). The sequence changes in one siRNA strand were compensated in the complementary siRNA strand to avoid perturbing the base-paired siRNA duplex structure. The sequence of all 2-nt 3' overhangs was TT (T, 2'-deoxythymidine) to reduce costs of synthesis. The TT/TT refe-

- 49 -

rence siRNA duplex was comparable in RNAi to the wild-type siRNA duplex AA/UG (Figure 17). The ability to mediate reporter mRNA destruction was quantified using the translation-based luminescence assay. Duplexes of siRNAs with inverted sequence segments showed dramatically reduced ability for targeting the firefly luciferase reporter (Figure 18). The sequence changes located between the 3' end and the middle of the antisense siRNA completely abolished target RNA recognition, but mutations near the 5' end of the antisense siRNA exhibit a small degree of silencing. Transversion of the A/U base pair located directly opposite of the predicted target RNA cleavage site, or one nucleotide further away from the predicted site, prevented target RNA cleavage, therefore indicating that single mutation within the centre of a siRNA duplex discriminate between mismatched targets.

3.3 Discussion

siRNAs are valuable reagents for inactivation of gene expression, not only in insect cells, but also in mammalian cells, with a great potential for therapeutic application. We have systematically analysed the structural determinants of siRNA duplexes required to promote efficient target RNA degradation in *D. melanogaster* embryo lysate, thus providing rules for the design of most potent siRNA duplexes. A perfect siRNA duplex is able to silence gene expression with an efficiency comparable to a 500 bp dsRNA, given that comparable quantities of total RNA are used.

3.4 The siRNA user guide

Efficiently silencing siRNA duplexes are preferably composed of 21-nt antisense siRNAs, and should be selected to form a 19 bp double helix with 2-nt 3' overhanging ends. 2'-deoxy substitutions of the 2-nt 3' overhanging ribonucleotides do not affect RNAi, but help to reduce the costs of RNA synthesis and may enhance RNase resistance of siRNA duplexes. More extensive 2'-deoxy or 2'-O-methyl modifications, however, reduce

- 50 -

the ability of siRNAs to mediate RNAi, probably by interfering with protein association for siRNAP assembly.

Target recognition is a highly sequence-specific process, mediated by the
5 siRNA complementary to the target. The 3'-most nucleotide of the guide
siRNA does not contribute to specificity of target recognition, while the
penultimate nucleotide of the 3' overhang affects target RNA cleavage, and
a mismatch reduces RNAi 2- to 4-fold. The 5' end of a guide siRNA also
10 appears more permissive for mismatched target RNA recognition when
compared to the 3' end. Nucleotides in the centre of the siRNA, located
opposite the target RNA cleavage site, are important specificity determi-
nants and even single nucleotide changes reduce RNAi to undetectable
level. This suggests that siRNA duplexes may be able to discriminate
15 mutant or polymorphic alleles in gene targeting experiments, which may
become an important feature for future therapeutic developments.

Sense and antisense siRNAs, when associated with the protein compo-
nents of the endonuclease complex or its commitment complex, were sug-
gested to play distinct roles; the relative orientation of the siRNA duplex in
20 this complex defines which strand can be used for target recognition.
Synthetic siRNA duplexes have dyad symmetry with respect to the double-
helical structure, but not with respect to sequence. The association of
siRNA duplexes with the RNAi proteins in the *D. melanogaster* lysate will
lead to formation of two asymmetric complexes. In such hypothetical
25 complexes, the chiral environment is distinct for sense and antisense
siRNA, hence their function. The prediction obviously does not apply to
palindromic siRNA sequences, or to RNAi proteins that could associate as
homodimers. To minimize sequence effects, which may affect the ratio of
sense and antisense-targeting siRNPs, we suggest to use siRNA sequences
30 with identical 3' overhanging sequences. We recommend to adjust the
sequence of the overhang of the sense siRNA to that of the antisense 3'
overhang, because the sense siRNA does not have a target in typical

- 51 -

knock-down experiments. Asymmetry in reconstitution of sense and anti-sense-cleaving siRNPs could be (partially) responsible for the variation in RNAi efficiency observed for various 21-nt siRNA duplexes with 2-nt 3' overhangs used in this study (Figure 14). Alternatively, the nucleotide
5 sequence at the target site and/or the accessibility of the target RNA structure may be responsible for the variation in efficiency for these siRNA duplexes.

References

Bass, B. L. (2000). Double-stranded RNA as a template for gene silencing. *Cell* 101, 235-238.

5

Bosher, J. M., and Labouesse, M. (2000). RNA interference: genetic wand and genetic watchdog. *Nat. Cell Biol.* 2, E31-36.

Caplen, N. J., Fleenor, J., Fire, A., and Morgan, R. A. (2000). dsRNA-mediated gene silencing in cultured *Drosophila* cells: a tissue culture model for the analysis of RNA interference. *Gene* 252, 95-105.

10

Catalanotto, C., Azzalin, G., Macino, G., and Cogoni, C. (2000). Gene silencing in worms and fungi. *Nature* 404, 245.

15

Chanfreau, G., Buckle, M., and Jacquier, A. (2000). Recognition of a conserved class of RNA tetraloops by *Saccharomyces cerevisiae* RNase III. *Proc. Natl. Acad. Sci. USA* 97, 3142-3147.

Clemens, M. J. (1997). PKR--a protein kinase regulated by double-stranded RNA. *Int. J. Biochem. Cell Biol.* 29, 945-949.

20

Cogoni, C., and Macino, G. (1999). Homology-dependent gene silencing in plants and fungi: a number of variations on the same theme. *Curr. Opin. Microbiol.* 2, 657-662.

25

Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D. C. (2000). An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* 101, 543-553.

30

- 53 -

Dernburg, A. F., Zalevsky, J., Colaiacovo, M. P., and Villeneuve, A. M. (2000). Transgene-mediated cosuppression in the *C. elegans* germ line. *Genes & Dev.* 14, 1578-1583.

- 5 Dunn, J. J. (1982). Ribonuclease III. In *The enzymes*, vol 15, part B, P. D. Boyer, ed. (New York: Academic Press), pp. 485-499.

Filippov, V., Solovyev, V., Filippova, M., and Gill, S. S. (2000). A novel type of RNase III family proteins in eukaryotes. *Gene* 245, 213-221.

10

Fire, A. (1999). RNA-triggered gene silencing. *Trends Genet.* 15, 358-363.

- Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806-811.

15

Grishok, A., Tabara, H., and Mello, C. C. (2000). Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* 287, 2494-2497.

- 20 Hamilton, A. J., and Baulcombe, D. C. (1999). A species of small anti-sense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950-952.

- Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 404, 293-296.

25

- Jacobsen, S. E., Running, M. P., and M., M. E. (1999). Disruption of an RNA helicase/RNase III gene in *Arabidopsis* causes unregulated cell division in floral meristems. *Development* 126, 5231-5243.

30

- 54 -

Jensen, S., Gassama, M. P., and Heidmann, T. (1999). Taming of transposable elements by homology-dependent gene silencing. *Nat. Genet.* 21, 209-212.

5 Kehlenbach, R. H., Dickmanns, A. & Gerace, L. (1998). Nucleocytoplasmic shuttling factors including Ran and CRM1 mediate nuclear export of NFAT In vitro. *J. Cell Biol.* 141, 863-874.

10 Kennerdell, J. R., and Carthew, R. W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* 95, 1017-1026.

15 Ketting, R. F., Haverkamp, T. H., van Luenen, H. G., and Plasterk, R. H. (1999). Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* 99, 133-141.

20 Ketting, R. F., and Plasterk, R. H. (2000). A genetic link between co-suppression and RNA interference in *C. elegans*. *Nature* 404, 296-298.

Lucy, A. P., Guo, H. S., Li, W. X., and Ding, S. W. (2000). Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. *EMBO J.* 19, 1672-1680.

25 Matsuda, S., Ichigotani, Y., Okuda, T., Irimura, T., Nakatsugawa, S., and Hamaguchi, M. (2000). Molecular cloning and characterization of a novel human gene (HERNA) which encodes a putative RNA-helicase. *Biochim. Biophys. Acta* 31, 1-2.

30 Milligan, J.F., and Uhlenbeck, O.C. (1989). Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol.* 180, 51-62.

- 55 -

- Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J. B., Jouette, D., Lacombe, A. M., Nikic, S., Picault, N., Remoue, K., Sanial, M., Vo, T. A., and Vaucheret, H. (2000). Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* 101, 533-542.
- Ngo, H., Tschudi, C., Gull, K., and Ullu, E. (1998). Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. USA* 95, 14687-14692.
- Nicholson, A. W. (1999). Function, mechanism and regulation of bacterial ribonucleases. *FEMS Microbiol. Rev.* 23, 371-390.
- Oelgeschlager, M., Larrain, J., Geissert, D., and De Robertis, E. M. (2000). The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling. *Nature* 405, 757-763.
- Pan, T., and Uhlenbeck, O. C. (1992). In vitro selection of RNAs that undergo autolytic cleavage with Pb^{2+} . *Biochemistry* 31, 3887-3895.
- Pelissier, T., and Wassenegger, M. (2000). A DNA target of 30 bp is sufficient for RNA-directed methylation. *RNA* 6, 55-65.
- Plasterk, R. H., and Ketting, R. F. (2000). The silence of the genes. *Curr. Opin. Genet. Dev.* 10, 562-567.
- Ratcliff, F. G., MacFarlane, S. A., and Baulcombe, D. C. (1999). Gene Silencing without DNA. RNA-mediated cross-protection between viruses. *Plant Cell* 11, 1207-1216.
- Robertson, H. D. (1990). *Escherichia coli* ribonuclease III. *Methods Enzymol.* 181, 189-202.

- 56 -

Robertson, H. D. (1982). *Escherichia coli* ribonuclease III cleavage sites. *Cell* 30, 669-672.

Romaniuk, E., McLaughlin, L. W., Neilson, T., and Romaniuk, P. J. (1982).
5 The effect of acceptor oligoribonucleotide sequence on the T4 RNA ligase
reaction. *Eur J Biochem* 125, 639-643.

Sharp, P. A. (1999). RNAi and double-strand RNA. *Genes & Dev.* 13, 139-
141.

10 Sijen, T., and Kooter, J. M. (2000). Post-transcriptional gene-silencing:
RNAs on the attack or on the defense? *Bioessays* 22, 520-531.

Smardon, A., Spoerke, J., Stacey, S., Klein, M., Mackin, N., and Maine, E.
15 (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in
germ-line development and RNA interference in *C. elegans*. *Curr. Biol.* 10,
169-178.

20 Svoboda, P., Stein, P., Hayashi, H., and Schultz, R. M. (2000). Selective
reduction of dormant maternal mRNAs in mouse oocytes by RNA inter-
ference. *Development* 127, 4147-4156.

Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons,
L., Fire, A., and Mello, C. C. (1999). The *rde-1* gene, RNA interference,
25 and transposon silencing in *C. elegans*. *Cell* 99, 123-132.

Tuschl, T., Ng, M. M., Pieken, W., Benseler, F., and Eckstein, F. (1993).
Importance of exocyclic base functional groups of central core guanosines
for hammerhead ribozyme activity. *Biochemistry* 32, 11658-11668.

- 57 -

Tuschl, T., Sharp, P. A., and Bartel, D. P. (1998). Selection in vitro of novel ribozymes from a partially randomized U2 and U6 snRNA library. EMBO J. 17, 2637-2650.

- 5 Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P., and Sharp, P. A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. Genes & Dev. 13, 3191-3197.

- 10 Ui-Tei, K., Zenno, S., Miyata, Y. & Saigo, K. (2000). Sensitive assay of RNA interference in Drosophila and Chinese hamster cultured cells using firefly luciferase gene as target. FEBS Letters 479, 79-82.

Verma, S., and Eckstein, F. (1999). Modified oligonucleotides: Synthesis and strategy for users. Annu. Rev. Biochem. 67, 99-134.

- 15 Voinnet, O., Lederer, C., and Baulcombe, D. C. (2000). A viral movement protein prevents spread of the gene silencing signal in Nicotiana benthamiana. Cell 103, 157-167.

- 20 Wassenegger, M. (2000). RNA-directed DNA methylation. Plant Mol. Biol. 43, 203-220.

- 25 Wianny, F., and Zernicka-Goetz, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. Nat. Cell Biol. 2, 70-75.

Wu, H., Xu, H., Miraglia, L. J., and Crooke, S. T. (2000). Human RNase III is a 160 kDa Protein Involved in Preribosomal RNA Processing. J. Biol. Chem. 17, 17.

30

- 58 -

Yang, D., Lu, H. and Erickson, J.W. (2000) Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in drosophila embryos. *Curr. Biol.*, 10, 1191-1200.

- 5 Zamore, P. D., Tuschl, T., Sharp, P. A., and Bartel, D. P. (2000). RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101, 25-33.

- 10 Zhang, K., and Nicholson, A. W. (1997). Regulation of ribonuclease III processing by double-helical sequence antideterminants. *Proc. Natl. Acad. Sci. USA* 94, 13437-13441.

- 59 -

Claims

- 5 1. Isolated double-stranded RNA molecule, wherein each RNA strand has a length from 19-25 nucleotides, wherein said RNA molecule is capable of target-specific nucleic acid modifications.
2. The RNA molecule of claim 1 wherein at least one strand has a 3'-overhang from 1-5 nucleotides.
- 10 3. The RNA molecule of claim 1 or 2 capable of target-specific RNA interference and/or DNA methylation.
4. The RNA molecule of any one of claims 1-3, wherein each strand
15 has a length from 19-23, particularly from 20-22 nucleotides.
5. The RNA molecule of any one of claims 2-4, wherein the 3'-overhang is from 1-3 nucleotides.
- 20 6. The RNA molecule of any one of claims 2-5, wherein the 3'-overhang is stabilized against degradation.
7. The RNA molecule of any one of claims 1-6, which contains at least one modified nucleotide analogue.
- 25 8. The RNA molecule of claim 7, wherein the modified nucleotide analogue is selected from sugar- or backbone modified ribonucleotides.
9. The RNA molecule according to claim 7 or 8, wherein the nucleotide
30 analogue is a sugar-modified ribonucleotide, wherein the 2'-OH group is replaced by a group selected from H, OR, R, halo, SH, SR¹,

- 60 -

NH₂, NHR, NR₂ or CN, wherein R is C₁-C₆ alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I.

- 5 10. The RNA molecule of claim 7 or 8, wherein the nucleotide analogue is a backbone-modified ribonucleotide containing a phosphothioate group.
- 10 11. The RNA molecule of any one of claims 1-10, which has a sequence having an identity of at least 50 percent to a predetermined mRNA target molecule.
12. The RNA molecule of claim 11, wherein the identity is at least 70 percent.
- 15 13. A method of preparing a double-stranded RNA molecule of any one of claims 1-12 comprising the steps:
- (a) synthesizing two RNA strands each having a length from 19-25 nucleotides, wherein said RNA strands are capable of forming a double-stranded RNA molecule,
- 20 (b) combining the synthesized RNA strands under conditions, wherein a double-stranded RNA molecule is formed, which is capable of target-specific nucleic acid modifications.
- 25 14. The method of claim 13, wherein the RNA strands are chemically synthesized.
- 30 15. The method of claim 13, wherein the RNA strands are enzymatically synthesized.

- 61 -

16. A method of mediating target-specific nucleic acid modifications in a cell or an organism comprising the steps:
- (a) contacting said cell or organism with the double-stranded RNA molecule of any one of claims 1-12 under conditions wherein target-specific nucleic acid modifications can occur, and
 - (b) mediating a target-specific nucleic acid modification effected by the double-stranded RNA towards a target nucleic acid having a sequence portion substantially corresponding to the double-stranded RNA.
17. The method of claim 16, wherein the nucleic acid modification is RNA interference and/or DNA methylation.
18. The method of claim 16 and 17 wherein said contacting comprises introducing said double-stranded RNA molecule into a target cell in which the target-specific nucleic acid modification can occur.
19. The method of claim 18 wherein the introducing comprises a carrier-mediated delivery or injection.
20. Use of the method of any one of claims 16-19 for determining the function of a gene in a cell or an organism.
21. Use of the method of any one of claims 16-19 for modulating the function of a gene in a cell or an organism.
22. The use of claim 20 or 21, wherein the gene is associated with a pathological condition.

- 62 -

23. The use of claim 22, wherein the gene is a pathogen-associated gene.
24. The use of claim 23, wherein the gene is a viral gene.
- 5 25. The use of claim 22, wherein the gene is a tumor-associated gene.
26. The use of claim 22, wherein the gene is an autoimmune disease-associated gene.
- 10 27. Pharmaceutical composition containing as an active agent at least one double-stranded RNA molecule of any one of claims 1-12 and a pharmaceutical carrier.
- 15 28. The composition of claim 27 for diagnostic applications.
29. The composition of claim 27 for therapeutic applications.
- 20 30. A eukaryotic cell or a eukaryotic non-human organism exhibiting a target gene-specific knockout phenotype wherein said cell or organism is transfected with at least one double-stranded RNA molecule capable of inhibiting the expression of an endogenous target gene or with a DNA encoding at least one double-stranded RNA molecule capable of inhibiting the expression of at least one
- 25 31. The cell or organism of claim 30 which is a mammalian cell.
32. The cell or organism of claim 31 which is a human cell.
- 30 33. The cell or organism of any one of claims 30-32 which is further transfected with at least one exogenous target nucleic acid coding

- 63 -

for the target protein or a variant or mutated form of the target protein, wherein said exogenous target nucleic acid differs from the endogenous target gene on the nucleic acid level such that the expression of the exogenous target nucleic acid is substantially less inhibited by the double stranded RNA molecule than the expression of the endogenous target gene.

34. The cell or organism of claim 33 wherein the exogenous target nucleic acid is fused to a further nucleic acid sequence encoding a detectable peptide or polypeptide.
35. Use of the cell or organism of any of claims 30-34 for analytic procedures.
36. The use of claim 35 for the analysis of gene expression profiles.
37. The use of claim 35 for a proteome analysis.
38. The use of any one of claims 35-37 wherein an analysis of a variant or mutant form of the target protein encoded by an exogenous target nucleic acid is carried out.
39. The use of claim 38 for identifying functional domains of the target protein.
40. The use of any one of claims 35-39 wherein a comparison of at least two cells or organisms is carried out selected from:
- (i) a control cell or control organism without target gene inhibition,
 - (ii) a cell or organism with target gene inhibition and
 - (iii) a cell or organism with target gene inhibition plus target gene complementation by an exogenous target nucleic acid.

- 64 -

41. The use of any one of claims 35-40 wherein the analysis comprises a functional and/or phenotypic analysis.
42. Use of a cell of any one of claims 30-34 for preparative procedures.
- 5 43. The use of claim 41 for the isolation of proteins or protein complexes from eukaryotic cells.
- 10 44. The use of claim 43 for the isolation of high molecular weight protein complexes which may optionally contain nucleic acids.
45. The use of any one of claims 35-44 in a procedure for identifying and/or characterizing pharmacological agents.
- 15 46. A system for identifying and/or characterizing a pharmacological agent acting on at least one target protein comprising:
- (a) a eukaryotic cell or a eukaryotic non-human organism capable of expressing at least one target gene coding for said at least one target protein,
 - 20 (b) at least one double-stranded RNA molecule capable of inhibiting the expression of said at least one endogenous target gene, and
 - (c) a test substance or a collection of test substances wherein pharmacological properties of said test substance or said
 - 25 collection are to be identified and/or characterized.
47. The system of claim 46 further comprising:
- (d) at least one exogenous target nucleic acid coding for the target protein or a variant or mutated from of the target
 - 30 protein wherein said exogenous target nucleic acid differs from the endogenous target gene on the nucleic acid level such that the expression of the exogenous target nucleic

- 65 -

acid is substantially less inhibited by the double stranded RNA molecule than the expression of the endogeneous target gene.

FIGURE 1A

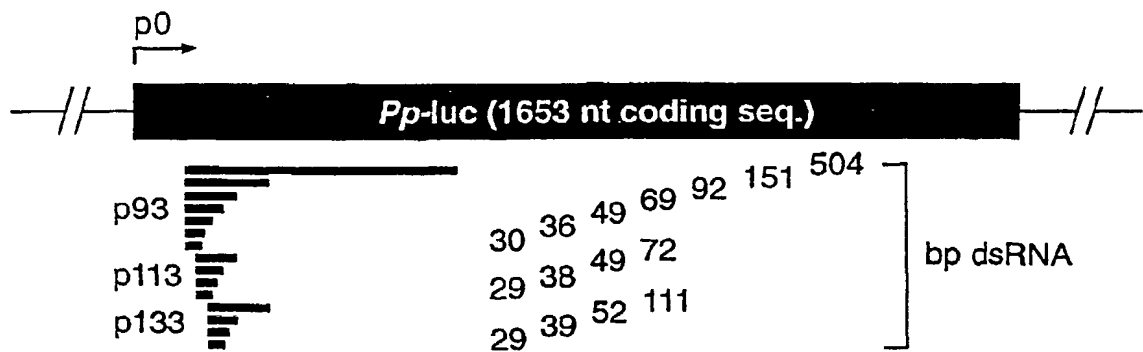


FIGURE 1B

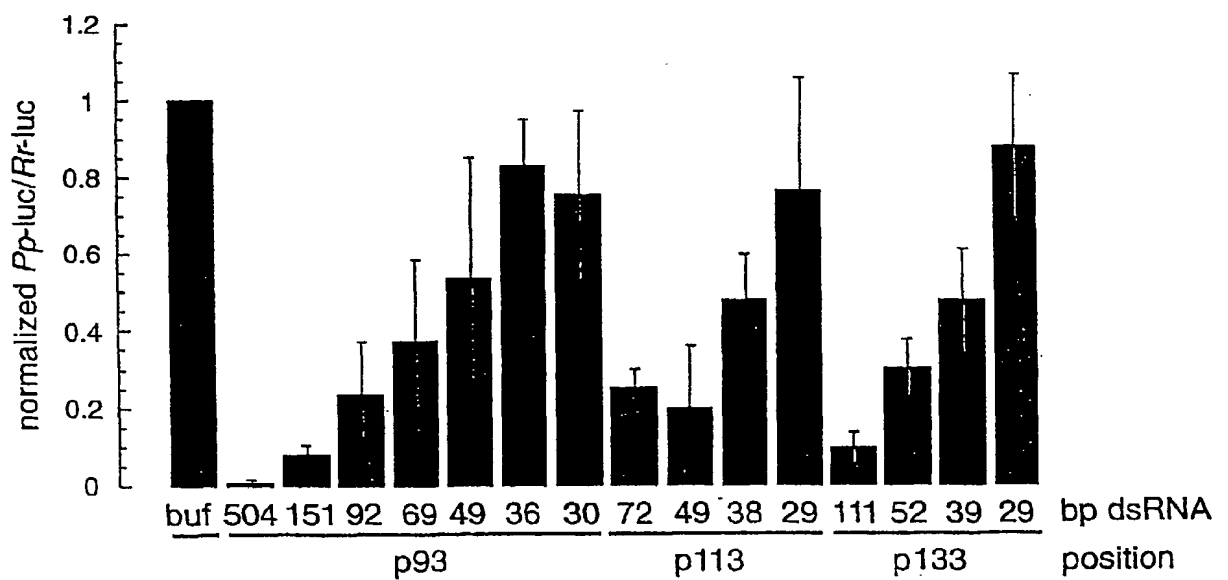


FIGURE 2

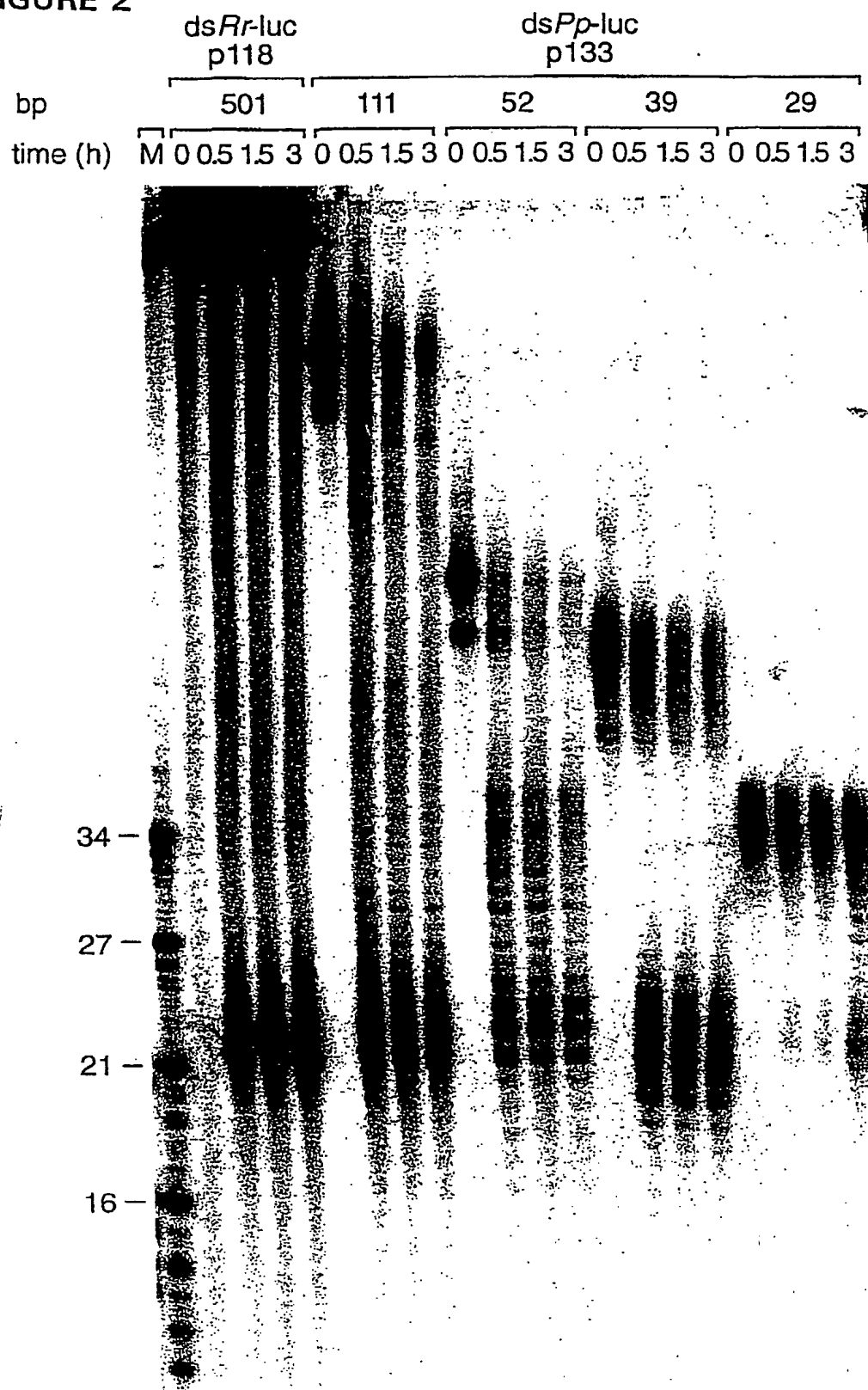


Figure 3A

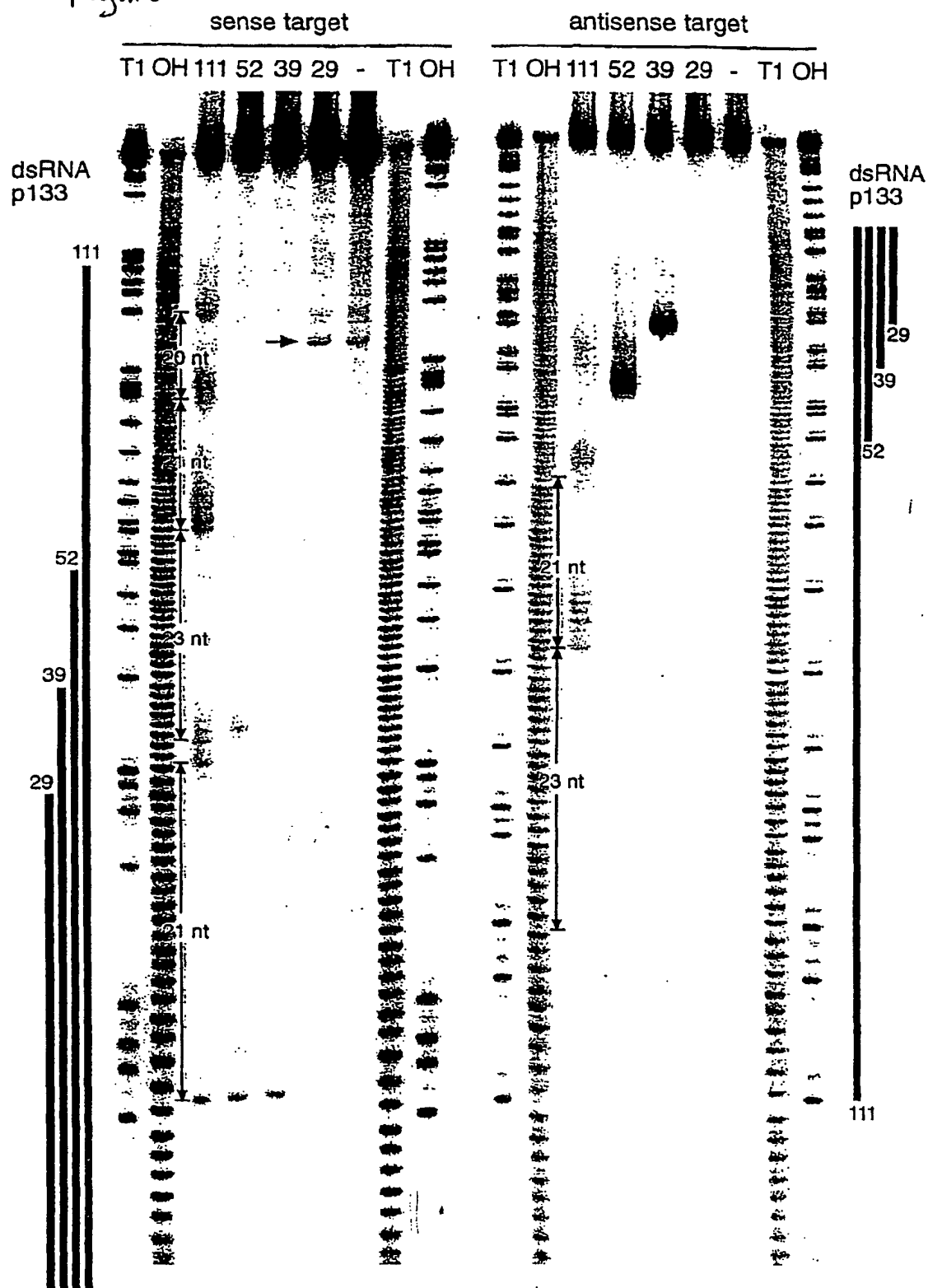


FIGURE 3B

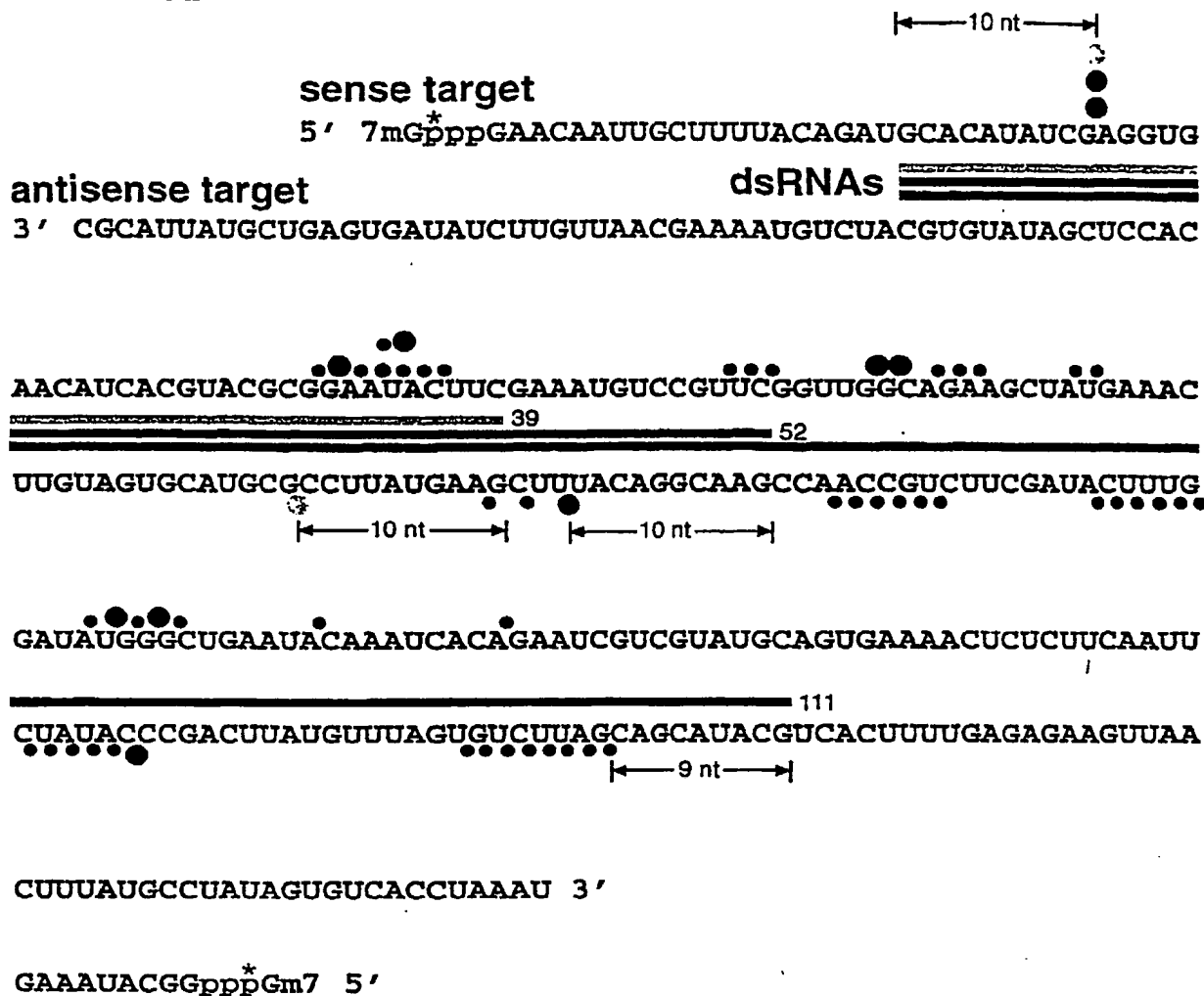


FIGURE 4A

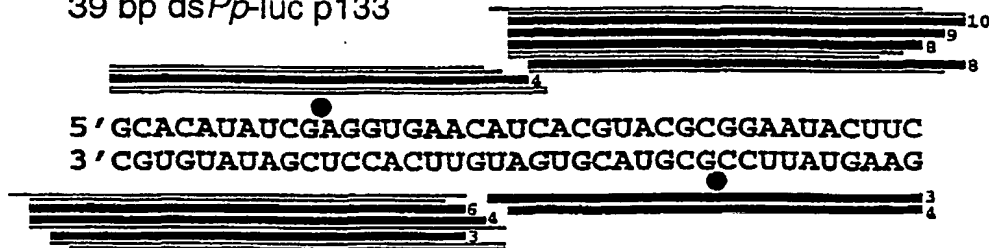
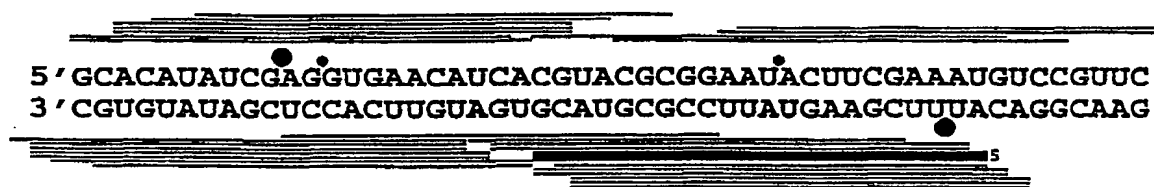
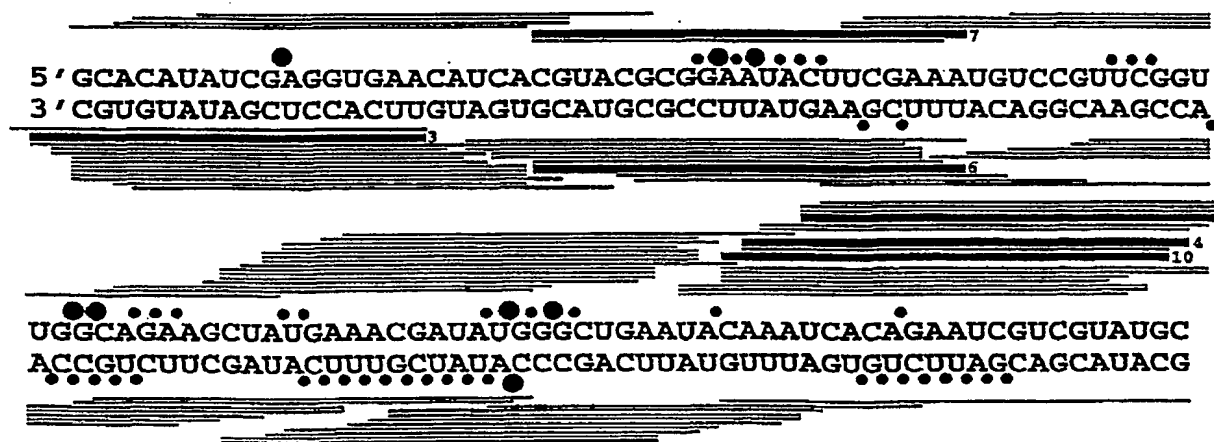
39 bp ds*Pp*-luc p13352 bp ds*Pp*-luc p133111 bp ds*Pp*-luc p133

FIGURE 4B

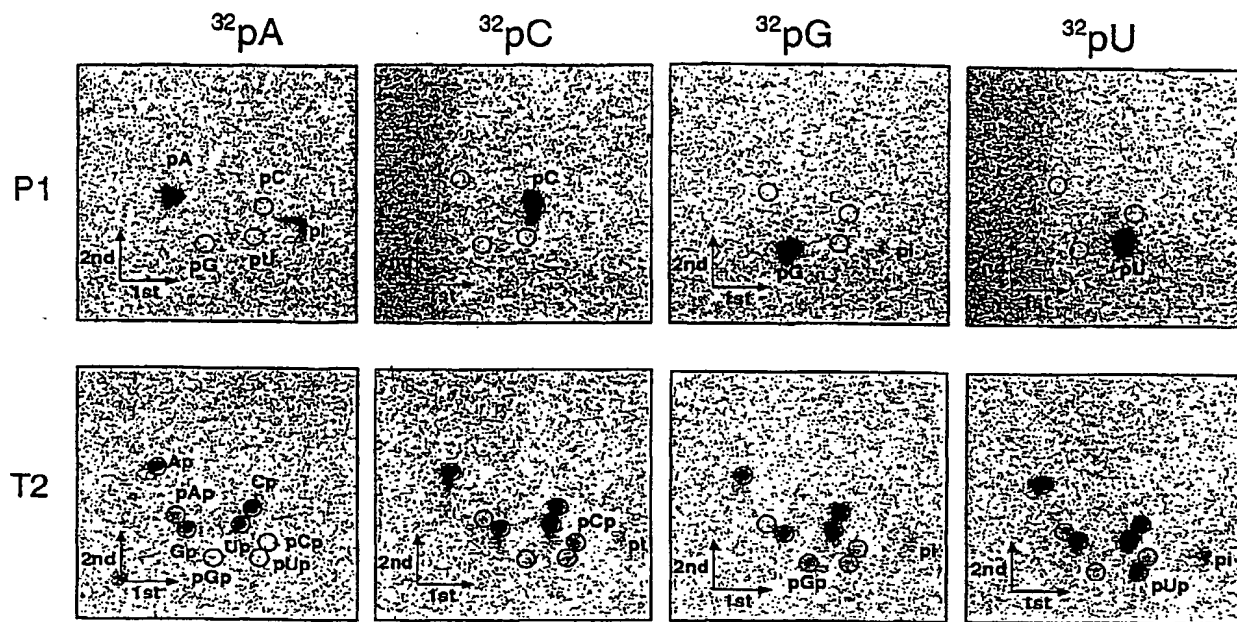


Figure 5A

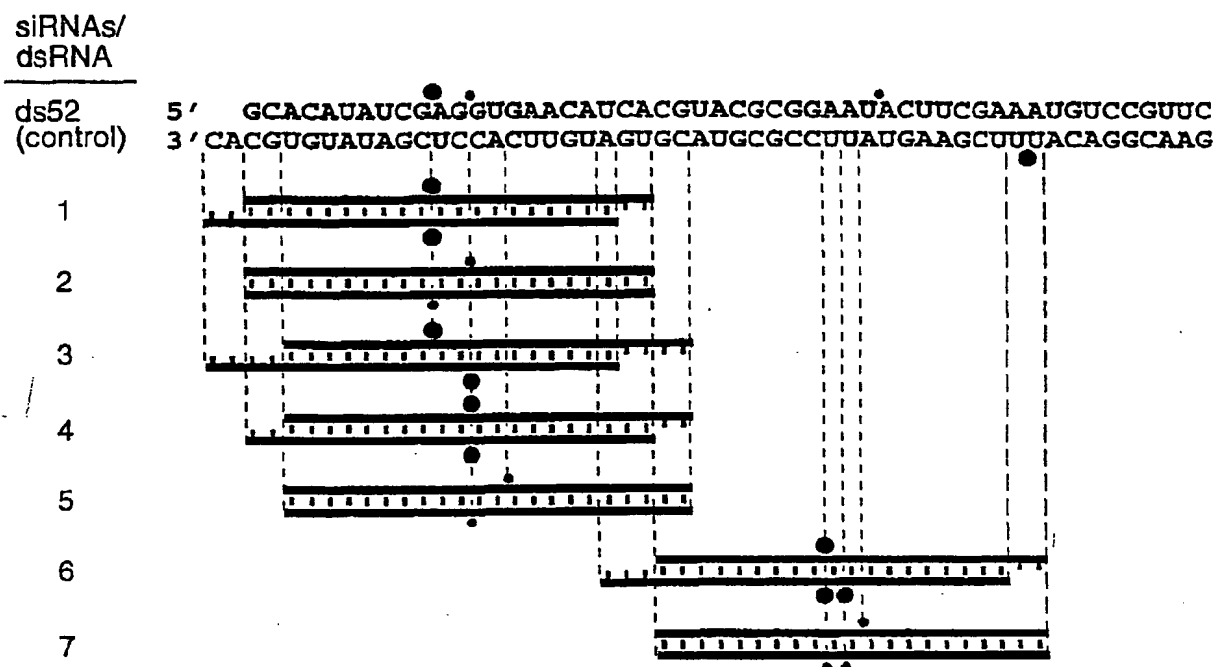


FIGURE 5B

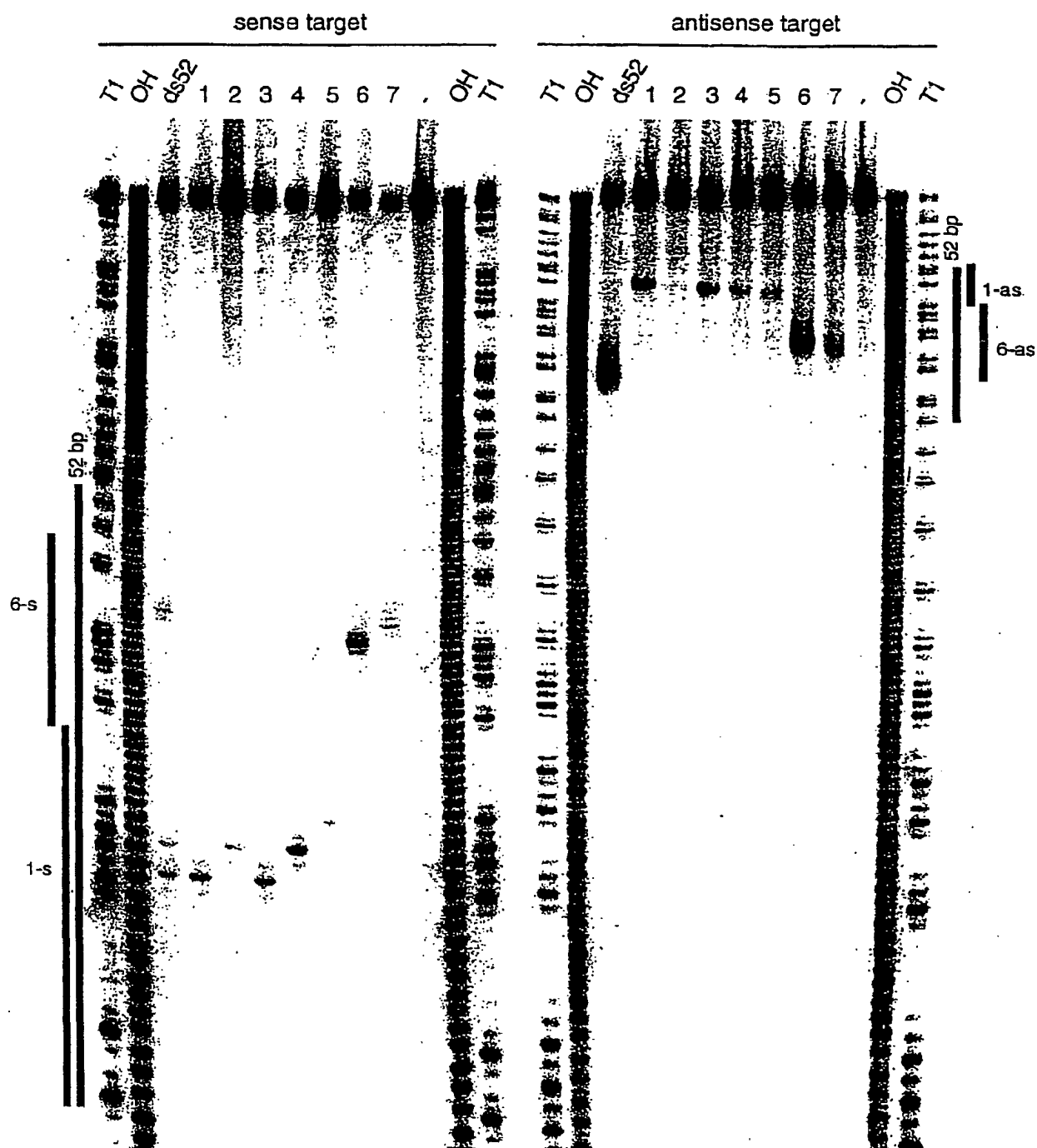


FIGURE 6A

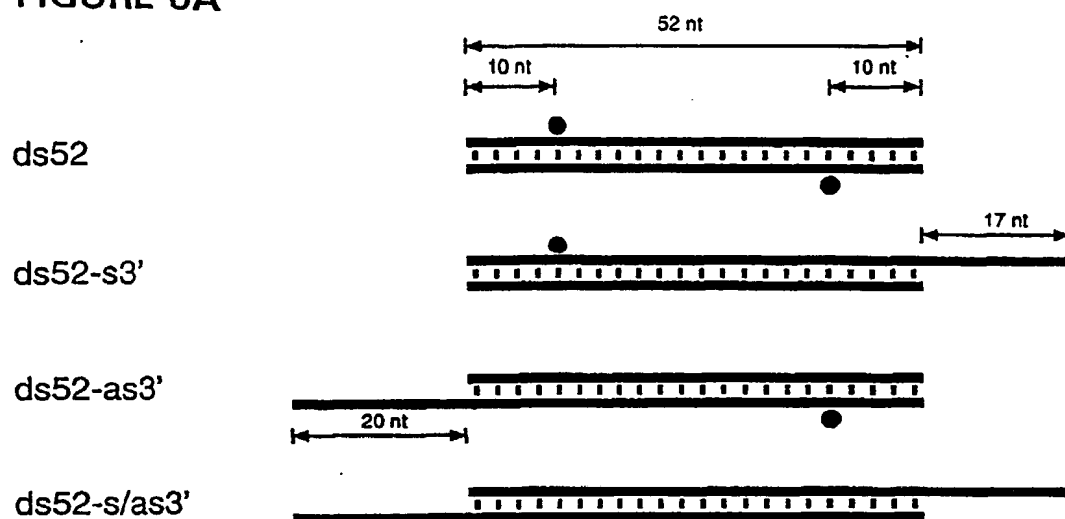
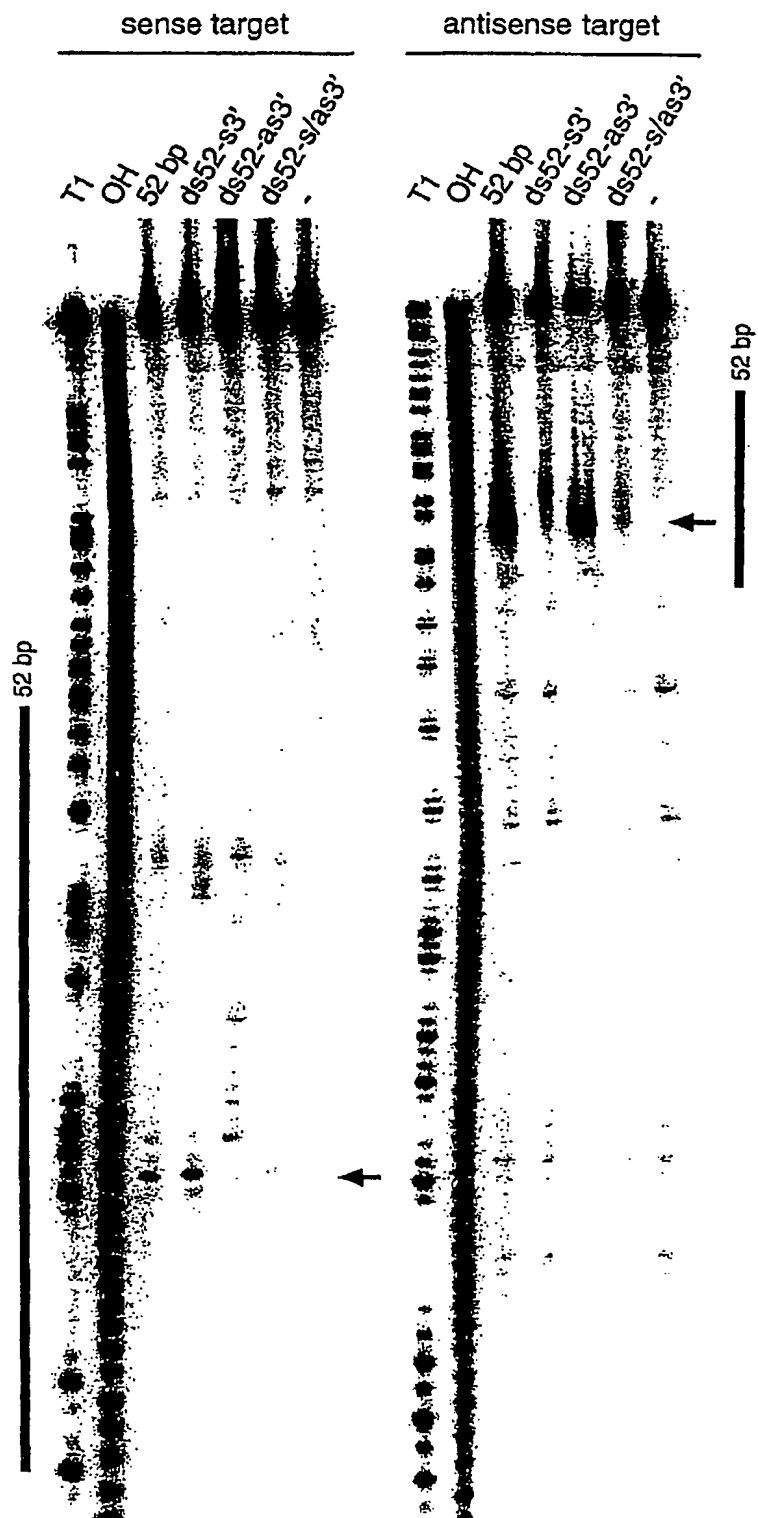


FIGURE 6B



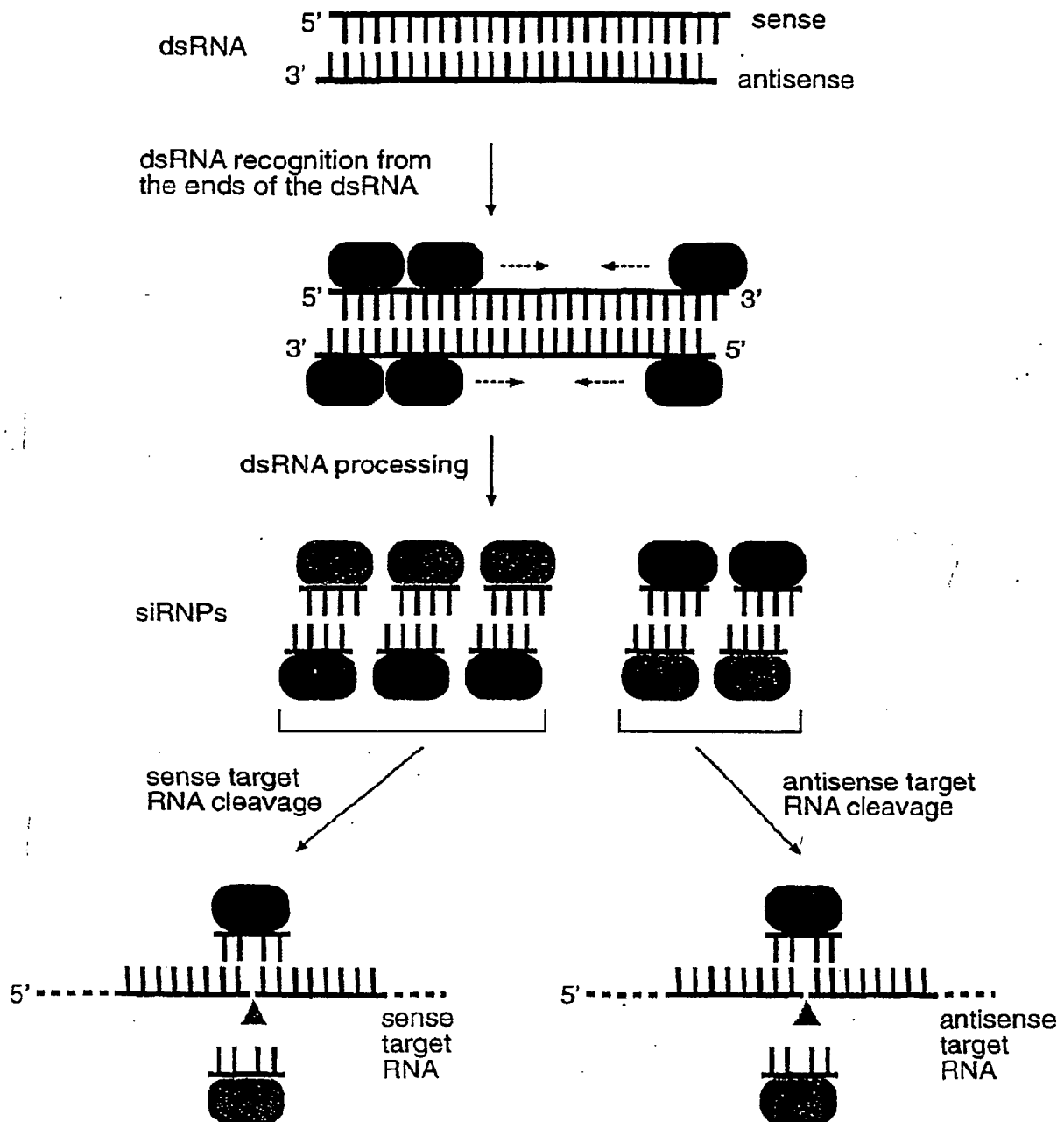
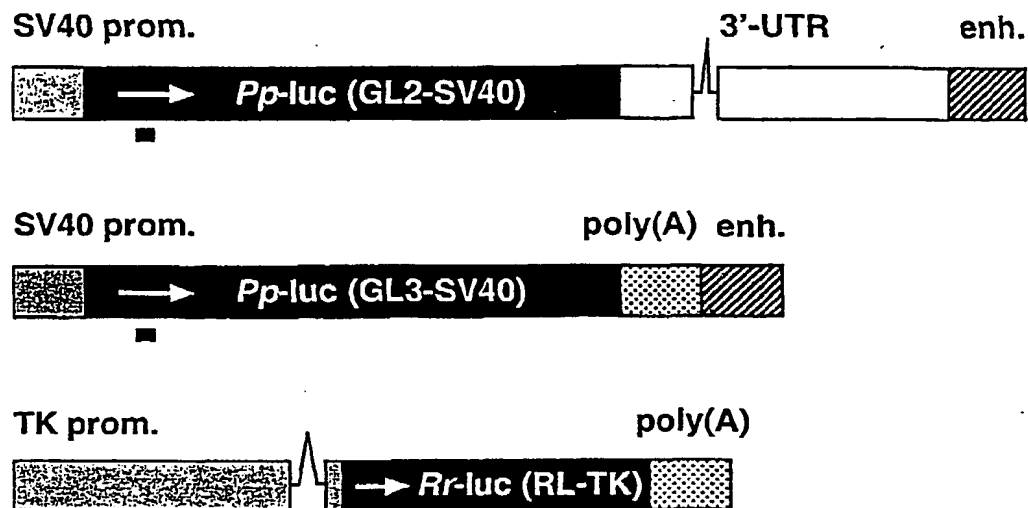


Figure 7

a



b

siRNA
duplex

uGL2	5' CGUACGCGGAUACUUCGAUU UUGCAUGCGCCUUAUGAAGCU 5'
GL2	5' CGUACGCGGAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5'
GL3	5' CUUACGCGAGUACUUCGATT TTGAAUGCGACUUAUGAAGCU 5'
invGL2	5' AGCUUCAUAAGGCGCAUGCTT TTUCGAAGUAUCCGCGUACG 5'
RL	5' AAACAUGCAGAAAAUGCUGTT TTUUUGUACGUCUUUUACGAC 5'

Figure 8

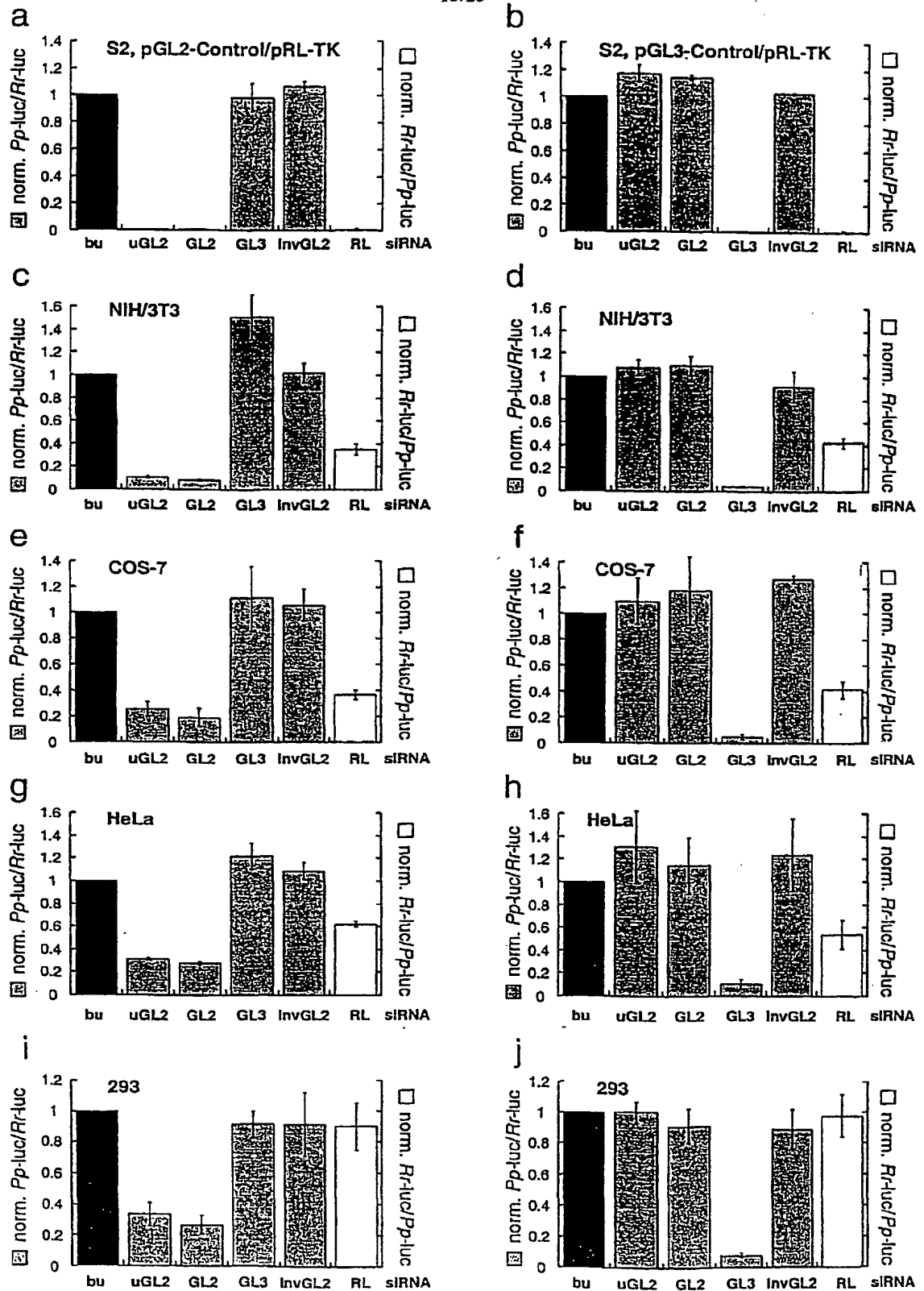


Figure 9

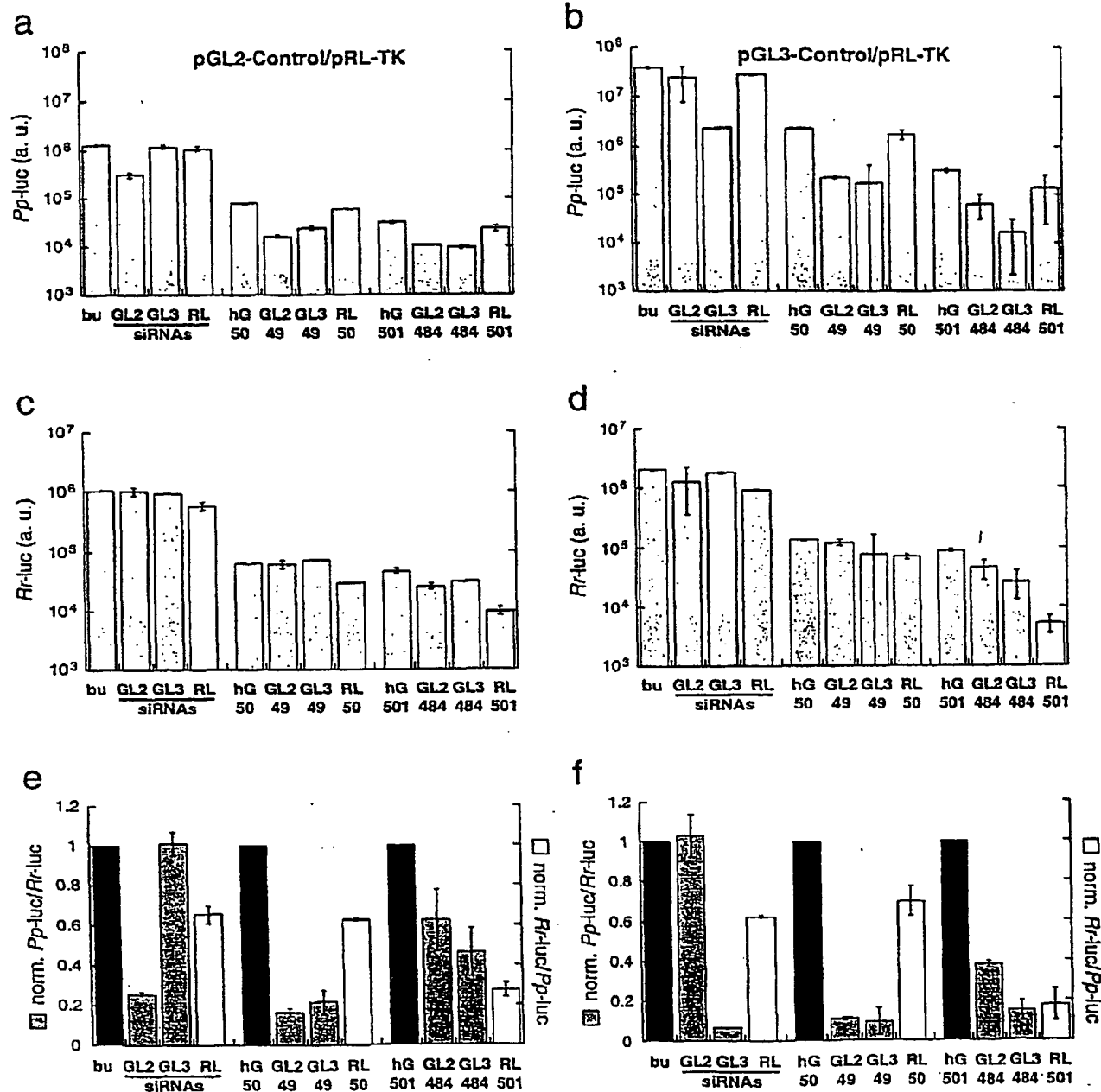
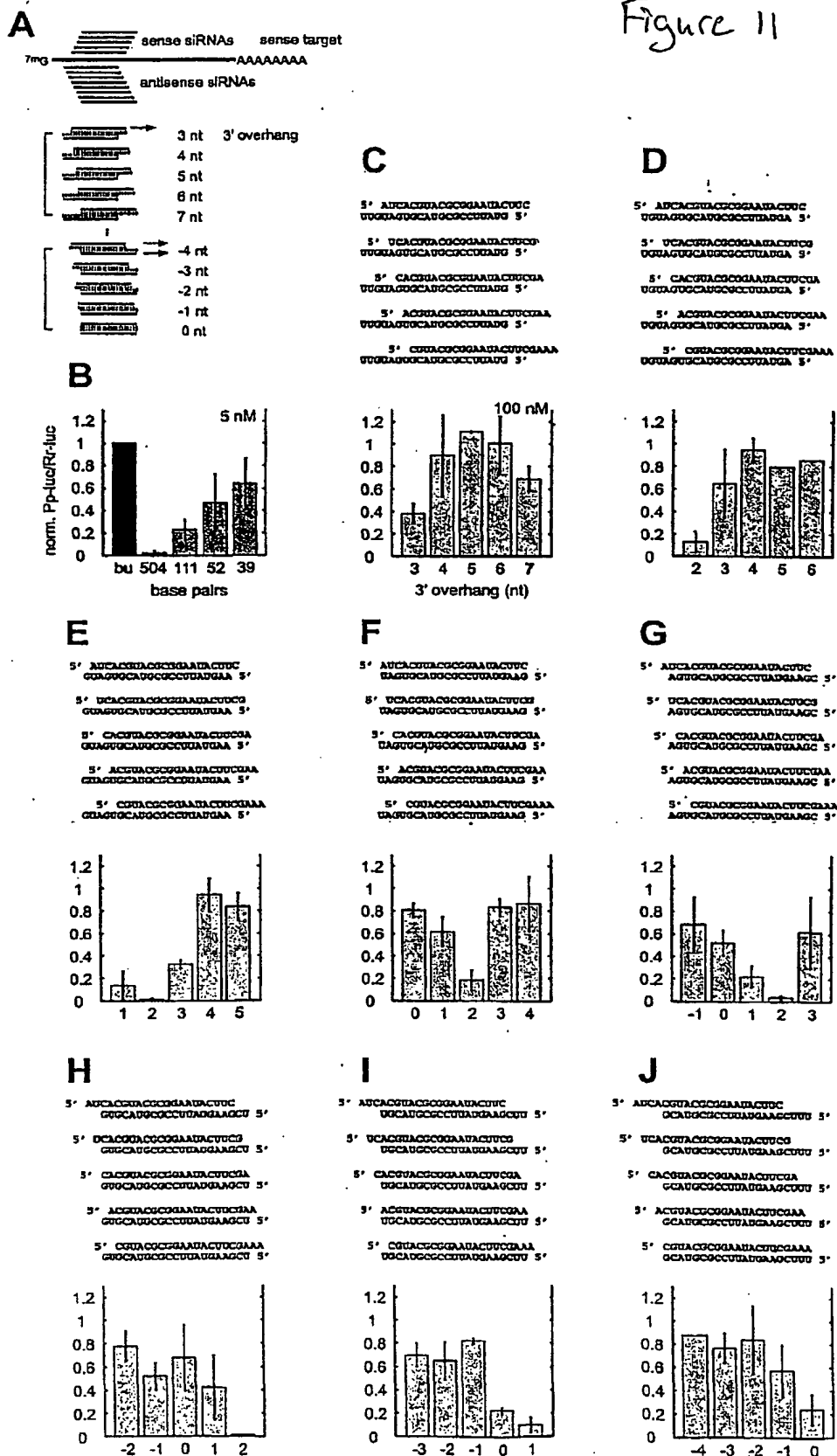


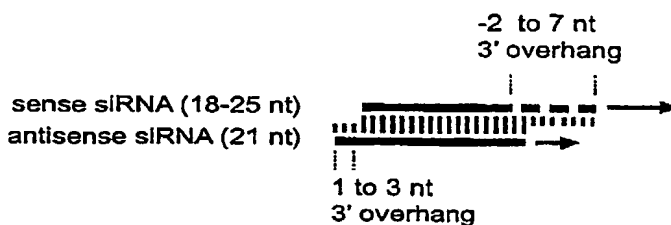
Figure 10

Figure 11



16/23

16/23

A**B**

5' CGUACGCGGAUAUACUUCG
UGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGA
UGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAA
UGCAUGCGCCUUAUGAAGCU 5'

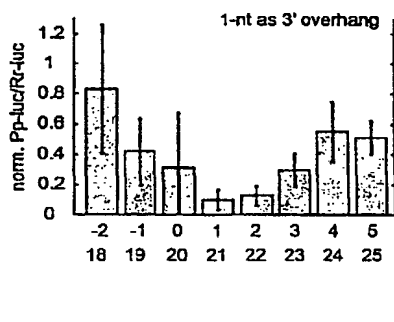
5' CGUACGCGGAUAUACUUCGAAA
UGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAAU
UGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAAUUG
UGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAAUUGU
UGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAAUUGUC
UGCAUGCGCCUUAUGAAGCU 5'

**C**

5' CGUACGCGGAUAUACUUCG
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGA
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAA
GUGCAUGCGCCUUAUGAAGCU 5'

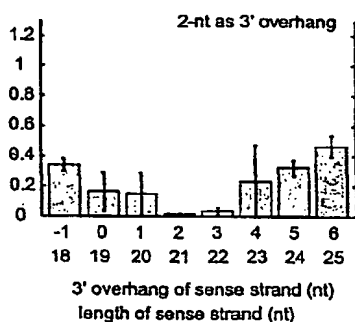
5' CGUACGCGGAUAUACUUCGAAA
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAAU
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAAUUG
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAAUUGU
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAAUUGUC
GUGCAUGCGCCUUAUGAAGCU 5'

**D**

5' CGUACGCGGAUAUACUUCG
AGUGCAUGCGCCUUAUGAAGC 5'

5' CGUACGCGGAUAUACUUCGA
AGUGCAUGCGCCUUAUGAAGC 5'

5' CGUACGCGGAUAUACUUCGAA
AGUGCAUGCGCCUUAUGAAGC 5'

5' CGUACGCGGAUAUACUUCGAAA
AGUGCAUGCGCCUUAUGAAGC 5'

5' CGUACGCGGAUAUACUUCGAAAU
AGUGCAUGCGCCUUAUGAAGC 5'

5' CGUACGCGGAUAUACUUCGAAAUUG
AGUGCAUGCGCCUUAUGAAGC 5'

5' CGUACGCGGAUAUACUUCGAAAUUGU
AGUGCAUGCGCCUUAUGAAGC 5'

5' CGUACGCGGAUAUACUUCGAAAUUGUC
AGUGCAUGCGCCUUAUGAAGC 5'

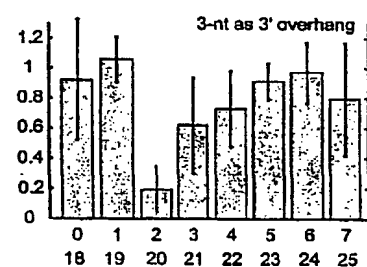
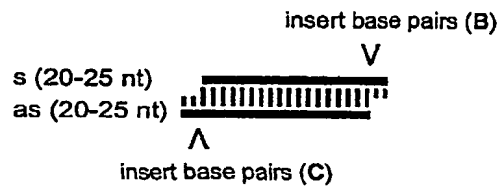


Fig. 13

A**B**

5' CGUACGCGGAUAUACUUCGAAA
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAA
GUGCAUGCGCCUUAUGAAGCU 5'

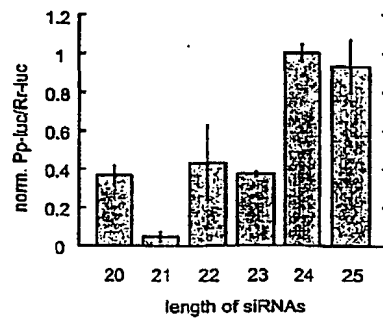
5' CGUACGCGGAUAUACUUCGAAA
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAA
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAA
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAA
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAA
GUGCAUGCGCCUUAUGAAGCU 5'

**C**

5' GUACGCGGAUAUACUUCGAAA
UGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAUAUACUUCGAAA
GUGCAUGCGCCUUAUGAAGCU 5'

5' ACGUACGCGGAUAUACUUCGAAA
AGUGCAUGCGCCUUAUGAAGCU 5'

5' CACGUACGCGGAUAUACUUCGAAA
UAGUGCAUGCGCCUUAUGAAGCU 5'

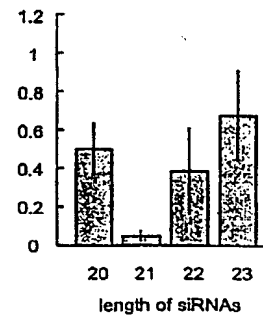


Fig. 14

S 5' CGUACGCGGAAUACUUCGAAA
as GUGCAUGCGCCUUAUGAAGCU 5'

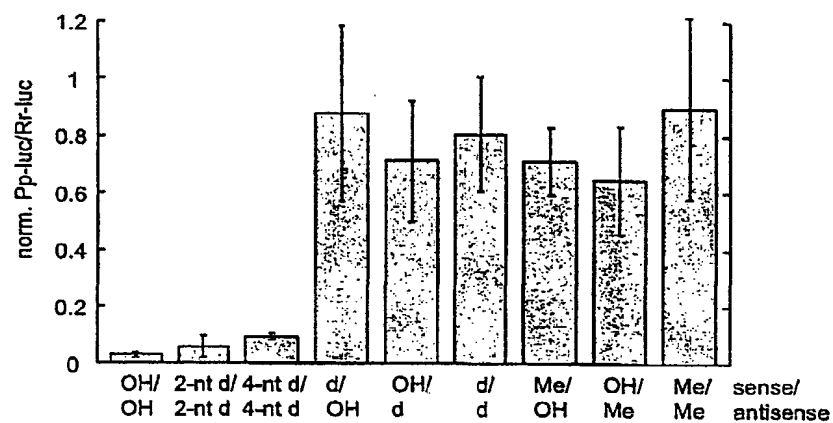


Fig. 15

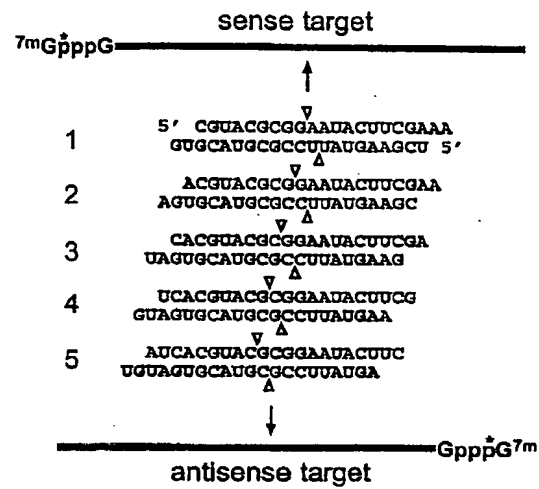
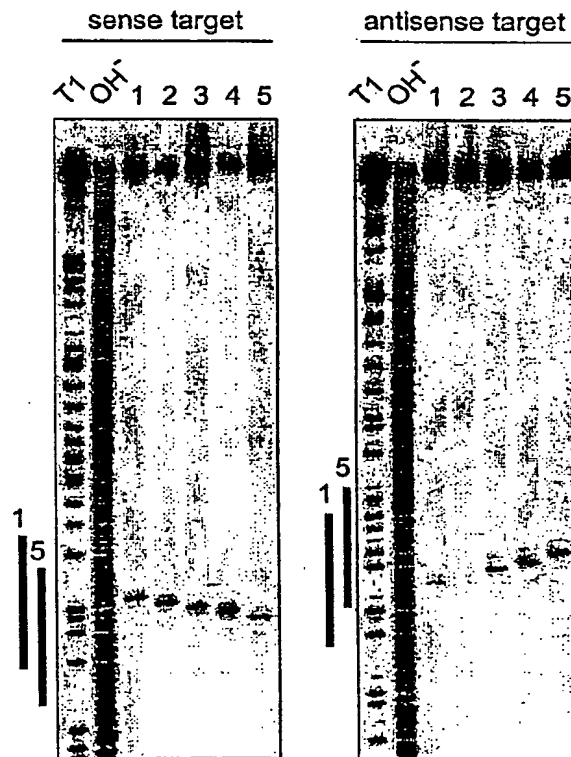
A**B**

Fig. 16

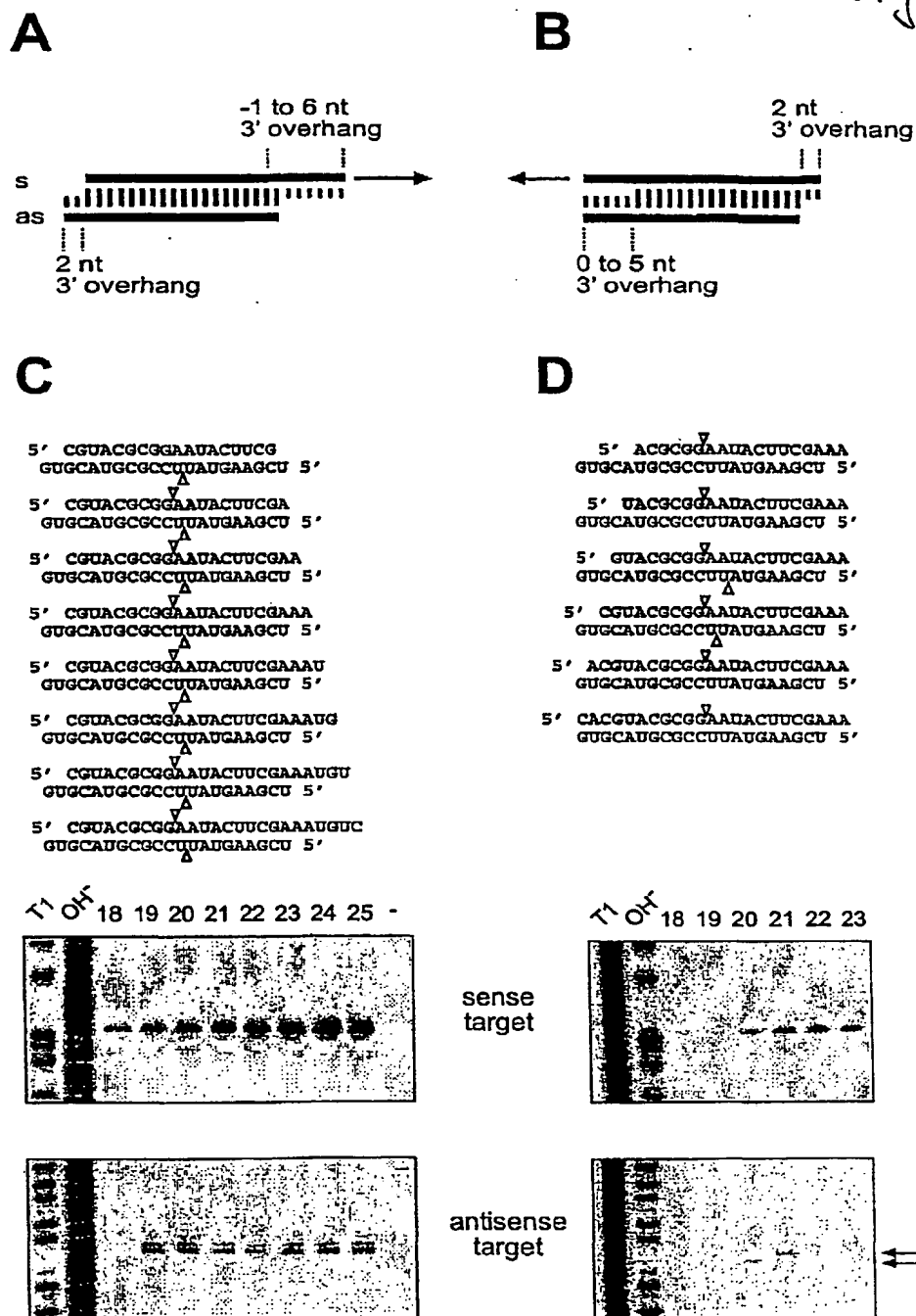


Fig. 17

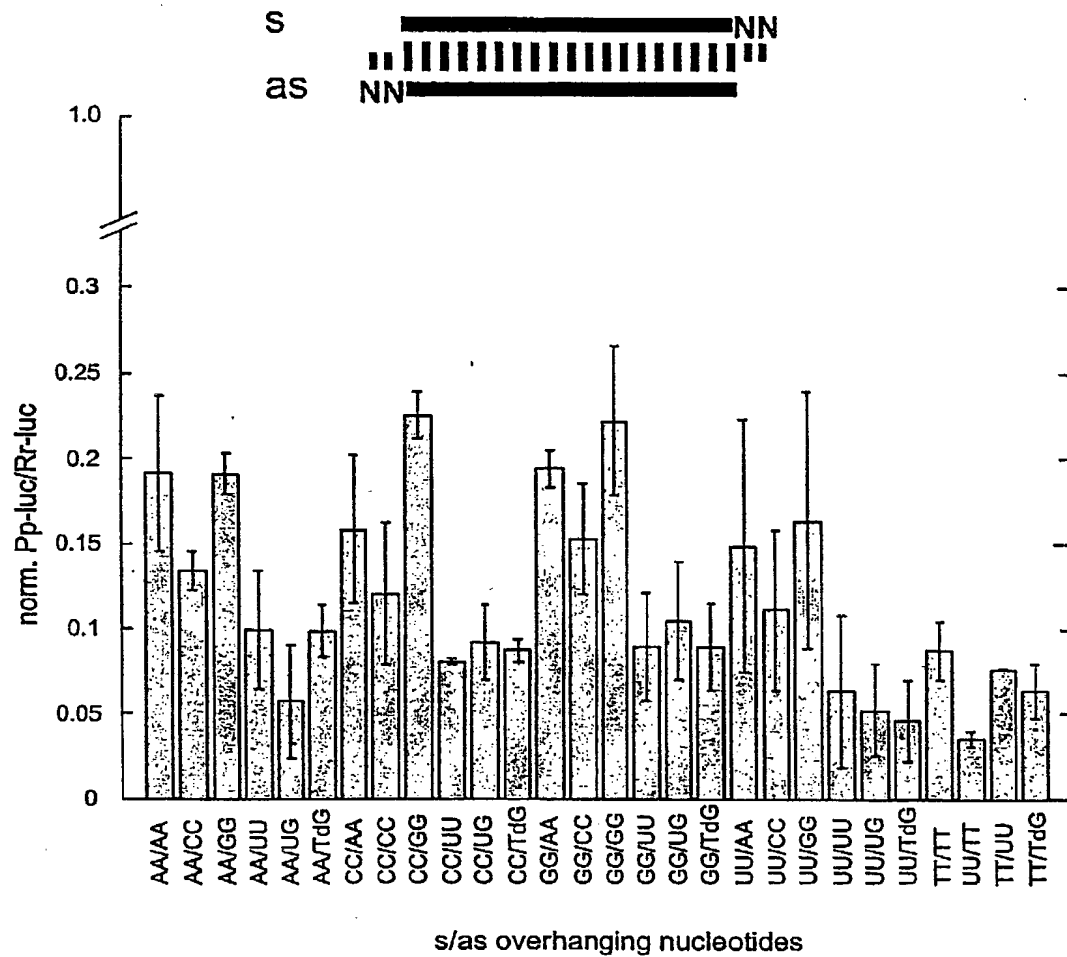


Fig. 18

ref	5' CGUACGCGGAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5'
1	5' AUGCGCGGAUACUUCGATT TTUACGCGCCUUAUGAAGCU 5'
2	5' CGUACGCGGAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5'
3	5' CGUACGCGGAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5'
4	5' CGUACGCGGAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5'
5	5' CGUACGCGGAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5'
6	5' CGUACGCGGAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5'
7	5' CGUACGCGGAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5'

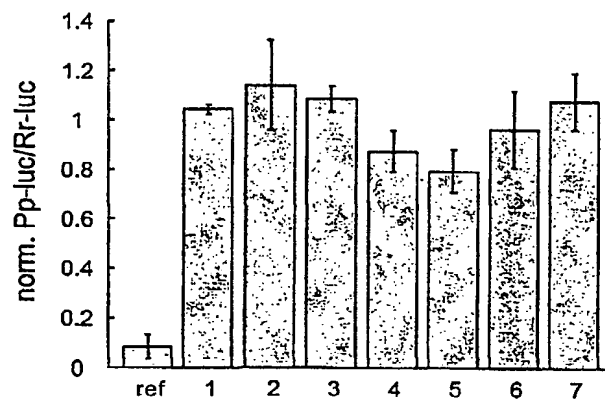


Figure 19

A

5' CGUACGCGGAAUACUUCGAA
GUGCAUGCGCCUUAUGAAGC 5'

5' CGUACGCGGAAUACUUCGAAA
GUGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAAUACUUCGATT
TTGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAAUACUUCGAAAU
GUGCAUGCGCCUUAUGAAGCUU 5'

5' CGUACGCGGAAUACUUCGAAAUG
GUGCAUGCGCCUUAUGAAGCUUU 5'

5' CGUACGCGGAAUACUUCGAAAUGU
GUGCAUGCGCCUUAUGAAGCUUUA 5'

5' CGUACGCGGAAUACUUCGAAAUGUC
GUGCAUGCGCCUUAUGAAGCUUUAC 5'

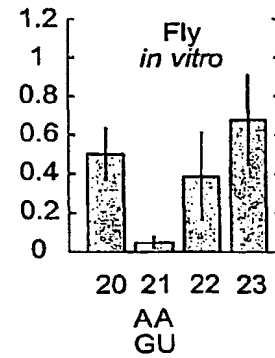
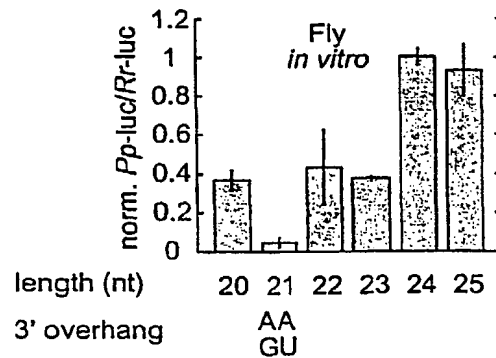
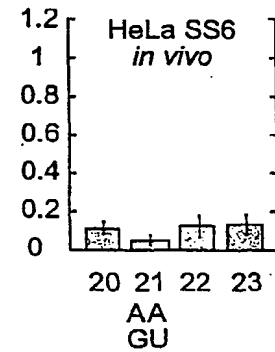
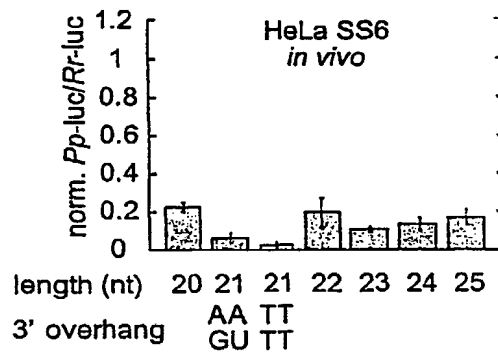
B

5' GUACGCGGAAUACUUCGAAA
UGCAUGCGCCUUAUGAAGCU 5'

5' CGUACGCGGAAUACUUCGAAA
GUGCAUGCGCCUUAUGAAGCU 5'

5' ACGUACGCGGAAUACUUCGAAA
AGUGCAUGCGCCUUAUGAAGCU 5'

5' CACGUACGCGGAAUACUUCGAAA
UAGUGCAUGCGCCUUAUGAAGCU 5'



**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.